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FORMATION *IN VITRO* OF LACTIC ACID AND BISULPHITE-BINDING SUBSTANCES* IN BRAIN AND OTHER TISSUES

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EXPERIMENTAL results are reported here on the amount of lactic acid and its formation in various nervous tissues (brain cortex slices from guinea pigs and rats; pig and pigeon retina), in muscle (rat diaphragm) and in guinea pig liver slices. In these experiments the colorimetric method for lactic acid estimation (BARKER and SUMMERSON, 1941) was compared with the ceric sulphate iodometric method of GORDON and QUASTEL (1939), as modified by WINNICK (1942) and described by CONWAY (1947).

As will be shown, similar results are obtained by both methods when fresh tissues are used. However, the results differ greatly for tissues which have been subjected to partial autolysis by incubating for several hours at 37°C with or without substrate; the values obtained with the modified ceric sulphate-iodometric method under certain conditions are many times greater than those obtained by the colorimetric method.

METHODS

Preparation of tissues. Brain cortex slices of guinea pigs or rats were prepared by hand as described by DEUTSCH (1936), the tissues to be sliced being placed on an ice-cold mount. Adherent saline was removed from the slices by repeated contact with a matt glass slide; the slices were then weighed to 0·1 mg and transferred to Warburg vessels containing 4 ml of ice-cold suspending medium. Pig retina was prepared as described by TERNER, EGGLESTON and KREBS (1950). To obtain pigeon retinas, the birds were decapitated, the eye-balls removed and the retinas rapidly isolated; the medium in the Warburg vessels was not previously cooled. Rat diaphragm was prepared by the method of RIESSER (1947), as described by KLEINZELLER (1954).

Experimental procedure. Each experiment was performed in three Warburg vessels with the following contents: vessel no. 1: 4 ml Krebs phosphate saline and 1 ml of 50% (w/v) trichloroacetic acid (TCA); vessel no. 2: 4 ml Krebs phosphate saline (without added substrate); vessel no. 3: 4 ml Krebs phosphate saline containing 10 mM-glucose/g.

Vessels containing the tissue were attached to manometers and, unless otherwise stated, filled with oxygen and incubated for 4 hr at 37° at a high shaking speed. Vessel no. 1, which contained TCA before introducing the tissue, was not incubated. After incubation the vessels were removed and 1 ml 50% TCA was added. From all the contents of the vessels an extract was prepared as described previously (VRBA, FOLBERGROVÁ and KANTŮREK, 1957), final volume 12 ml (10% TCA w/v). Of each extract 4 ml were transferred to centrifuge tubes, and the following additions were made: 4 ml H_2O, 0·5 ml 20% (w/v) $CuSO_4.5H_2O$ and 0·5 g solid $Ca(OH)_2$. After thorough mixing of the contents for 1 hr, the tubes were centrifuged and the supernatant filtered (filtrate *A*). In some cases filtrate *A* was prepared by adding 1·5 ml $CuSO_4$ solution and 1·5 g solid $Ca(OH)_2$ to 12 ml of the TCA extract. For each experimental series (two, four or six groups of three Warburg flasks) filtrate *A* was also prepared as described above for a series of standard solutions of lithium lactate in 10% TCA. The colorimetric and iodometric estimations of lactic acid were then carried out on these filtrates.

Colorimetric estimation of lactic acid. Filtrate *A* (0·25 ml) was added to 6 ml of ice-cold conc.

* The term 'bisulphite-binding substances' denotes here those substances which on oxidation with ceric sulphate at room temperature yield products specifically absorbed by a solution of sodium bisulphite.

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sulphuric acid and lactic acid was then estimated by the method of BARKER and SUMMERSON (1941); see also UMBREIT, BURRIS and STAUFER (1951). Two parallel estimations were always carried out; these agreed within 10 per cent.

Ceric sulphate-iodometric estimation of lactic acid. Filtrate *A* (2 ml) and 1 ml of a saturated solution of ceric sulphate in 2 N-H_2SO_4 were placed into the outer chamber of a standard Conway dish. Into the centre well 2 ml of a 0·25 N-solution of sodium bisulphite were pipetted. After closing the Conway dish, the contents of the outer chamber were mixed; oxidation and diffusion proceeded overnight at room temperature (20–25°). The contents of the centre well were then transferred to a 50 ml Erlenmeyer flask, the centre well was rinsed three times with 2 ml of distilled water and these washings were also added to the Erlenmeyer flask. Starch solution was then added and excess bisulphite titrated with 0·1 N-iodine; after exact equivalence had been obtained (i.e. when a change of colour was obtained on adding 0·05 ml of 0·003 N-iodine or 0·003 N-thiosulphate), an excess of solid Na_2HPO_4 was added to the flask and after several minutes the solution was titrated with 0·003 N-iodine. Each estimation was carried out in duplicate; the difference between duplicates did not exceed ±5 per cent. In the same way filtrate *A* of standard lactate solution was analysed.

In some cases lactic acid was also estimated in portions of filtrate *A* by oxidation with permanganate at 100° (LIEB and ZACHERL, 1932).

Some further details of the methods used are presented in the Results section.

RESULTS AND DISCUSSION

Representative results are given in Table 1. It will be seen from the typical experiment no. 3 that in fresh guinea pig brain cortex, deproteinized with TCA without incubation, 5·1 μmoles lactic acid/g tissue were found by the colorimetric method, and 4·6 μmoles/g by the ceric sulphate iodometric method. These results are very similar. After a 4 hr incubation of the tissue in phosphate saline in the presence of glucose, 93·5 μmoles/g lactic acid were found by the colorimetric procedure, but 915 μmoles/g by the ceric sulphate-iodometric method. Thus in this particular experiment the value obtained by the ceric sulphate-iodometric method exceeded by nearly ten times the value obtained by the colorimetric procedure. This result suggests that during aerobic incubation of brain cortex slices in glucose-containing saline, bisulphite-binding substances other than lactic acid, are formed. These substances are also formed in the absence of added substrate when, owing to the small carbohydrate reserve of this tissue, no formation of lactic acid can occur. As shown in experiment no. 3, after a 4 hr incubation of the slices in saline without added glucose, 4·2 μmoles lactic acid were found by the colorimetric method, but 208 μmoles 'lactic acid' by the ceric sulphate procedure. Similar results were obtained with other nervous tissues, and also with muscle (expts. nos. 11 and 12) and liver (expts. nos 13 and 14). In several experiments $ZnSO_4$—NaOH had been tried as a protein precipitant according to WINNICK (1942). This procedure gave similar results to those obtained using TCA.

In experiments with pigeon retina more than 3000 μmoles 'lactic acid' were found per gram of tissue by the ceric sulphate method; if this represented true lactic acid, its weight would exceed that of the solids of the incubated tissue. These high values suggest that the bisulphite-binding substance(s), formed on oxidation of the metabolic products of incubated tissues with ceric sulphate, require considerably more iodine after decomposition of the bisulphite complex than the theoretical requirement if this substance were acetaldehyde.

Experiments nos. 9 and 10 in Table 1 show that the formation of bisulphite-binding substance(s) in surviving nervous tissue also takes place under anaerobic conditions. Data presented in Table 2 show that the formation of bisulphite-binding

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TABLE 1—COMPARISON OF LACTIC ACID ESTIMATIONS IN FRESH AND INCUBATED TISSUES BY THE COLORIMETRIC AND CERIC SULPHATE-IODOMETRIC PROCEDURES

Contents of vessels: 100–120 mg of brain cortex slices or pig retina, or 20–30 mg pigeon retina, 4 ml phosphate–saline. Unless otherwise stated, tissues were incubated at 37° for 4 hr with oxygen as gas phase. The results are expressed in μmoles 'lactic acid'/g fresh weight. Upper figures were obtained by the colorimetric procedure, lower figures (italics) by the modified ceric sulphate method.

No. of experiment	Protocol	Tissue	Initial values	Saline without substrate	Saline with 10 m-moles/l. glucose
1	19a	Pig retina	6·6	20·8	117·0
			17·3	*363·0*	*788·0*
2	19b	Pig retina	6·9	16·7	108·5
			9·0	*461·0*	*501·0*
3	20a	Guinea pig brain cortex slices	5·1	4·2	93·5
			4·6	*208·0*	*915·0*
4	20b	Guinea pig brain cortex slices	18·1	13·5	78·2
			14·3	*605·0*	*588·0*
5*	22e	Pigeon retina	47·5	57·0	708·0
			44·5	*2780·0*	*2110·0*
6*	22f	Pigeon retina	55·0	30·9	795·0
			74·2	*2910·0*	*2640·0*
7†	22a	Pigeon retina	36·4	44·7	233·0
			49·5	*1900·0*	*3330·0*
8†	22b	Pigeon retina	40·9	36·7	314·0
			39·2	*2840·0*	*3420·0*
9‡	22c	Pigeon retina	78·4	34·1	324·0
			58·5	*1210·0*	*873·0*
10‡	22d	Pigeon retina	59·0	35·5	376·0
			—	*870·0*	*910·0*
11	29a	Rat diaphragm	24·3	13·6	20·0
			37·2	*402·0*	*511·0*
12	29b	Rat diaphragm	15·4	15·0	20·6
			30·1	*630·0*	*450·0*
13	30d	Guinea pig liver slices	2·8	8·4	10·2
			10·3	*1510·0*	*544·0*
14	30e	Guinea pig liver slices	3·0	8·1	8·7
			19·4	*1045·0*	*819·0*

* Air as gas phase.
† Air as gaseous phase, 1 hr incubation.
‡ Incubation of 1 hr, nitrogen as gaseous phase. Traces of oxygen were removed by passing the gas through alkaline pyrogallol. [The low results of lactic acid in fresh tissue (first column) are subject to a considerable experimental error which, however, does not affect the argument presented.]

substance(s) by incubated nervous tissue is not inhibited by the presence of 10^{-2} M-NaF or 10^{-3} M-NaCN.

Results presented in Fig. 1 show that the formation of bisulphite-binding substance(s) increases with increasing ratio of medium to suspended tissue. The highest formation of these substances was observed with pigeon retina, where the smallest amount of tissue was used, and thus the ratio of volume of suspending medium to weight of suspended tissue was greatest.

TABLE 2.—THE EFFECT OF SODIUM FLUORIDE AND CYANIDE ON THE FORMATION OF BISULPHITE-BINDING SUBSTANCES IN SLICES OF GUINEA PIG BRAIN CORTEX

Each vessel contained 40–50 mg of slices, suspended in 4 ml saline without substrate. Incubated at 37° for 1 hr; air as gas phase. The results are expressed in μ moles 'lactic acid'/g, fresh weight. Each figure represents the mean of four experiments ± S.E.

Initial values	Inhibitors		
	0	10^{-2} M-NaF	10^{-3} M-NaCN
35·1 ± 5·8	1160 ± 94·5	1109 ± 120·6	1076 ± 142·6

The question arises whether the bisulphite-binding substance(s) are formed by an enzymic or non-enzymic process. Therefore the following experiment was performed. Fresh pig retina (1312 mg) was extracted three times with 12 ml 10% TCA. After

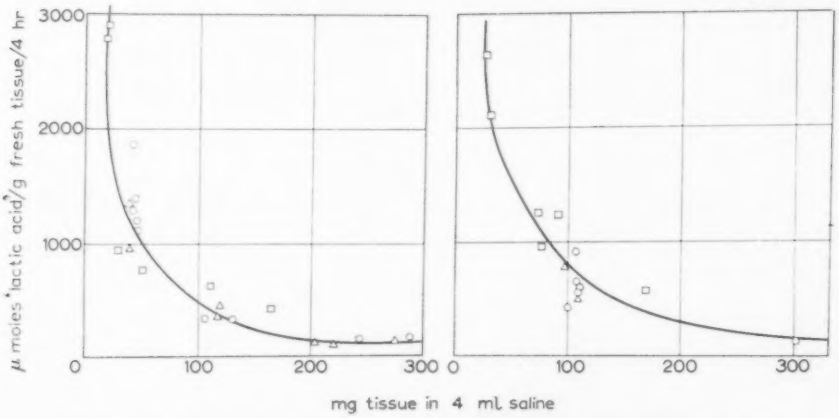

FIG. 1.—The effect of the ratio volume of medium/weight of tissue on the formation *in vitro* of bisulphite-binding substances in nervous tissue.
Each vessel contained 20–300 mg of tissue and 4 ml of phosphate saline without (left graph) or with substrate (right graph). Incubation at 37° for 4 hr, gas phase oxygen or air. For preparation of TCA extract and analytical methods see text. The results are expressed in μmoles 'lactic acid'/g fresh weight. □ pigeon retina; △ pig retina; ○ guinea pig brain cortex slices.

decanting the supernatant, the extracted tissue was homogenized with 1·5 g quartz powder and hydrolysed with 50 ml 20% (w/v) HCl on a water bath at 100° for 4 hr. The hydrochloric acid was then removed *in vacuo* and the dry solid dissolved in 10 ml 10% TCA. Of this solution 4 ml were then treated with $CuSO_4$ and $Ca(OH)_2$ as described in Methods. Using the ceric sulphate-iodometric method, 10·65 μmoles 'lactic acid' was found per gram tissue; this quantity is negligible compared with the amount of bisulphite-binding substance(s) formed in the course of tissue incubation. It thus appears that the formation of bisulphite-binding substance(s) is due to an enzymic process.

It is known that acetaldehyde reacts with the *p*-hydroxydiphenyl reagent (BARKER and SUMMERSON, 1941; KLENDSHOJ and FELDSTEIN, 1955). It has now been found that the bisulphite-binding substance(s) formed on oxidation of the metabolic products during incubation (autolysis) of nervous or muscle tissue resemble acetaldehyde

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not only by reacting with bisulphite, but also by reacting with the *p*-hydroxydiphenyl reagent.

Fresh slices of guinea pig brain cortex (40–50 mg) were placed in three Warburg vessels, each containing 4 ml of phosphate saline with or without substrate. To the first vessel 1 ml of 50% TCA was added at time T_0, and the second and third vessels were incubated for 1 hr at 37°. From the contents of both vessels a TCA extract of final volume 12 ml was prepared as described in Methods. After removal of carbohydrate, three 2 ml portions of the filtrate were placed in three Conway dishes and oxidized with ceric sulphate as described above. After complete oxidation, the contents of the centre well of the Conway dishes were transferred by means of pipettes into calibrated centrifuge tubes. The central chamber was rinsed with 2 ml of distilled

TABLE 3.—COLORIMETRIC AND IODOMETRIC ESTIMATION OF BISULPHITE-BINDING SUBSTANCES

Contents of vessels: 4 ml saline, 40–50 mg guinea pig brain cortex slices or 100–120 mg rat diaphragm. Brain slices incubated 1 hr at 37°, air as gas phase; diaphragm incubated 4 hr at 37° with oxygen as gas phase. Preparation of TCA extracts and details of analytical methods in text. The results are expressed in μ moles 'acetaldehyde'/g fresh weight of tissues ± S.E.

No. of expts.		Method	Initial values	Saline without substrate	Saline with 10 μmoles/l. glucose
6	Brain cortex	Colorimetric	<30	1137 ± 155·7	1239 ± 121·9
		Iodometric	<30	1201 ± 120·7	1162 ± 67
3	Diaphragm	Colorimetric	<30	467 ± 71·6	417 ± 51
		Iodometric	<30	481·7 ± 47·2	447·3 ± 37·7

water and this was added to the contents of the calibrated tube. Thus from the contents of each Warburg vessel 12 ml of a solution of bisulphite was prepared, in which were absorbed the oxidation products in the incubation and tissue. In 0·25 ml portions of this solution acetaldehyde was estimated by the method of BARKER and SUMMERSON, as described in Methods. In 1 ml portions of the same solutions acetaldehyde was estimated iodometrically. Each estimation was carried out in duplicate, and the experiment was repeated six times. The results of these experiments are presented in Table 3. In the same table the results of similar experiments with diaphragm are recorded. It may be seen that during incubation of tissues, even in the absence of substrate, substance(s) are formed which on oxidation yield a bisulphite-binding compound, which can then be estimated with the same result either iodometrically, or by the colorimetric test for acetaldehyde.

It has been shown by BARKER and SUMMERSON (1941), and also by a number of other authors that the colorimetric method for the estimation of lactic acid gives results identical with the chemical method, based on the oxidation of lactic acid with acid permanganate at 100° (FRIEDEMANN, COTONIO and SHAFFER, 1927; FRIEDEMANN and GRAESER, 1933; LIEB and ZACHERL, 1932). This fact does not contradict our observation that, under the above experimental conditions, ceric sulphate brings about the liberation of volatile bisulphite-binding substances from extracts of incubated tissues; these derive not only from lactic acid, but also from some other source(s). Typical experiments are recorded in Table 4, which demonstrate the

oxidizing capacity of ceric sulphate under the conditions described. In slices of brain cortex, incubated in absence of substrate, no formation of bisulphite-binding substance can be observed using the method of LIEB and ZACHERL (1932); on the other hand, a considerable formation of such substance(s) can be shown using the modified ceric sulphate method. The same amount of lactic acid formed by brain cortex slices, incubated in glucose-containing saline, was found using either the LIEB

TABLE 4.—COMPARISON OF THREE METHODS FOR THE ESTIMATION OF BISULPHITE–BINDING SUBSTANCES IN SLICES OF RAT BRAIN CORTEX

Each vessel contained 100–120 mg of slices and 4 ml of phosphate saline. Incubation at 37° for 4 hr; oxygen as gas phase. In portions of the TCA-extract, from which carbohydrates had been removed, lactic acid was estimated by three methods. The results are expressed in μmoles 'lactic acid'/g fresh weight.

Method	Experiment No. 29d			Experiment No. 29f		
	Initial values	Saline without substrate	Saline with 10 μmoles/l glucose	Initial values	Saline without substrate	Saline with 10 μmoles/l glucose
Colorimetric method (BARKER and SUMMERSON, 1941)	<10	<10	90·1	<10	<10	37·6
Oxidation with $KMnO_4$ (LIEB and ZACHERL, 1932)	<10	<10	89·0	<10	<10	33·1
Oxidation with ceric sulphate (CONWAY, 1947)	20·4	596·0	668·0	46·4	356·0	359·0

and ZACHERL and the colorimetric method; however, a considerably higher amount was found using the ceric sulphate method. Similar results were obtained using rat diaphragm.

The following conclusions can be drawn from these results. On incubation of nervous and other tissues, a substance (or substances) is formed which is not identical with lactic acid, and which, on oxidation with acid ceric sulphate at room temperature yields a compound in some respects similar to acetaldehyde. This compound is volatile; it forms with sodium bisulphite a complex, which is decomposed by di-sodium phosphate and it gives a colour reaction with *p*-hydroxydiphenyl of the same or a very similar type as acetaldehyde. The substance differs however from lactic acid in that it is not converted to acetaldehyde or to an acetaldehyde-like compound by concentrated sulphuric acid or acid permanganate at 100°.

In view of these results the values obtained by some authors using the ceric sulphate method for lactic acid determination may have to be reconsidered.

SUMMARY

The formation of lactic acid in slices of guinea pig and rat brain cortex, pig and pigeon retina, rat diaphragm and guinea pig liver slices was examined using a

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colorimetric method and a modified ceric sulphate—iodometric method. It was found that:

(1) When a trichloroacetic acid extract of fresh tissue (i.e. tissue deproteinized immediately after preparation) was analysed, both methods gave practically the same results.

(2) In TCA extracts of tissues that had been incubated for 1–4 hr in Krebs phosphate-saline in the presence of glucose, the values for lactic acid were several times higher using the ceric sulphate method than with the colorimetric method.

(3) In TCA extracts of nervous tissue that had been incubated for 1–4 hr in absence of substrate, practically no lactic acid was found by the colorimetric method. However, there was formed a considerable amount of a substance (or substances), which on oxidation with ceric sulphate was volatile, formed a complex with sodium bisulphite, and gave a colour reaction with *p*-hydroxydiphenyl.

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THE FLUORIMETRIC MEASUREMENT OF GLUTAMIC DECARBOXYLASE AND ITS DISTRIBUTION IN BRAIN*

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THE uniquely high activity of glutamic decarboxylase (GDC)† in brain has been reported (ROBERTS, 1950; WINGO and AWAPARA, 1950). ROBERTS (1956), using pooled tissues, has shown that there is a five- to six-fold greater activity of GDC in grey matter than in white matter of the cat brain. Because of the limitations of the manometric methods used, further resolution of the cytoarchitectonic distribution of GDC has awaited the development of more sensitive analytical methods for measuring the activity of this enzyme. It has been found that certain amino acids and indole derivatives, when allowed to react with ninhydrin at an alkaline pH‡, form highly fluorescent products. Of the substances tested, under the conditions to be described, γ-aminobutyric acid (γAB) has given the greatest amount of fluorescence and can be measured at a concentration of 10^{-7} M. A fluorescence method has been developed to measure the activity of GDC in as little as 3 μg dry weight of brain tissue.

The activity of this enzyme has been measured in various areas of the monkey and rabbit brain as well as in the whole brain of mouse and rat. The histochemical distribution of GDC in the layers of the monkey cerebellar cortex and selected white tracts of the rabbit and rat has also been determined. White matter has been shown to be extremely poor in GDC.

EXPERIMENTAL

Method for GDC

Material. The general techniques for obtaining suitable frozen-dried material of 0·1 to 5 μg dry weight from brain, and the general methods for manipulation and measurement of the small weights and volumes, have been described (LOWRY, 1953; LOWRY, ROBERTS, LEINER, WU, FARR and ALBERS, 1954; ROBINS and SMITH, 1953).

Reagents. All reagents were obtained from California Foundation for Biochemical Research or Mallinckrodt Chemical Works, A.R. grade reagents. They were prepared in glass-distilled water and

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† Abbreviations used in this paper are as follows: GDC = glutamic decarboxylase; γAB = γ-aminobutyric acid; GA = glutamic acid; TCA = trichloroacetic acid.

‡ PASIECKA and MORGAN (1956) observed that the blue colour formed by treating phenylalanine with ninhydrin on paper chromatograms was modified by subsequent spraying with alkali. They found an increased intensity and stability of the blue colour with a shift in its absorption peak. In this laboratory, the additional observation was made that following chromatography the phenylalanine spot after spraying with ninhydrin and alkali was fluorescent. Attempts to reproduce this fluorescent reaction in a test tube were successful and the reaction was then extended to other compounds. Whether or not the reaction in a test tube is the same as that on paper has not been ascertained.

stored frozen, unless otherwise indicated. Ninhydrin, 14 mM, made up in 0·5 M-sodium or potassium carbonate buffer pH 9·9 to 10·0.* When stored frozen, it was stable for at least 2 months. Standard solutions of γAB, 2 and 4 mM. Pyridoxal phosphate, 50 mM. Potassium phosphate buffer, 0·4 M, pH 6·4. Glutamic acid (GA), 100 mM, neutralized to pH 6·5 to 7·0. Trichloroacetic acid (TCA), 10 per cent, was stored in refrigerator. Copper tartrate reagent: 1·6 g Na_2CO_3, 329 mg tartaric acid, and 300 mg of $CuSO_4.5H_2O$ were dissolved in 1 l. of water, stored at room temp. In order to avoid precipitation, these three compounds were dissolved separately and added in the order mentioned to 900 ml of water and made up to volume. The complete buffer-substrate for the enzyme assay in homogenates was made up just before use by combining 1 ml of 0·4 M-phosphate buffer and 1 ml of 100 mM GA with 40 μl of 50 mM pyridoxal phosphate. When analysing frozen-dried tissues, which do not have an appreciable volume compared to the buffer-substrate, the buffer-substrate was diluted 1:1 with water. The final concentrations during enzyme incubation were GA 25 mM, phosphate buffer 0·1 M, and pyridoxal phosphate 0·5 mM.

Procedure. The frozen-dried tissue sample (3 to 30 μg) is placed in a special tube of 2·5 mm bore (Lowry *et al.*, 1954) in an ice bath and 1 μl of the 1:1 buffer-substrate mixture is added. (For homogenates and standards, an equal volume of the undiluted buffer-substrate solution is mixed with an equal volume of the homogenate or with an equal volume of the γAB standard and 1 μl is pipetted into similar tubes.) The Parafilm-capped tubes are incubated at 38° for exactly 60 min, returned to the ice bath, and to each is added 1 μl 10% TCA. The tubes are centrifuged at 3000 rev/min for 10 min and a 1 μl aliquot of the supernatant fluid is mixed with 2 μl of the ninhydrin reagent in special microfluorimeter tubes (Lowry *et al.*, 1954). The tubes are capped with tightly fitting cut-off vial stoppers, heated at 60° for 30 min, and 50 μl of copper tartrate reagent are then added. After 15 min, the tubes are read in the Farrand fluorimeter adapted for micro-tubes (Lowry *et al.*, 1954). A Corning glass filter 5860 is used in the primary and a combination of the 5543 and 3387 filters is used in the secondary.

Since there is an appreciable tissue blank, the blanks consist of the homogenate or another bit of frozen-dried tissue† to which the 1 μl of TCA is added before the buffer-substrate. It is then not necessary to add TCA to these blanks after the incubation.

When it is not necessary to use a micro procedure as described above, the enzyme activity in from 20 μg to 25 mg of dry brain may be conveniently assayed using commercially available apparatus. In a serological tube, 7 × 70 mm (Aloe Scientific, St. Louis, No. 78140B), the enzyme may be assayed in a 30–300 μl incubation volume and read in a final volume of 1 ml in commercially available tubes (10 × 75 mm, Corning no. 9820) which fit the Farrand fluorimeter. In this more macro-procedure it is important to maintain the ratio of 1 vol of TCA supernatant fluid to 2 vols of the ninhydrin reagent and to dilute the ninhydrin reaction product seventeen to twenty-five times with the copper tartrate reagent.

Comment on the enzyme method. The optimal conditions found for the enzyme assay are essentially those already described (Roberts and Frankel, 1951). The pH optimum for GDC is pH 6·4. There may be a secondary but smaller peak of activity at approximately pH 9. Optimal substrate concentration is 25 mM-GA and optimal pyridoxal phosphate concentration is 0·5 mM. There is a fall-off in activity of the enzyme with time in all of the species that we have examined (Fig. 1).§ This fall-off is less in the monkey than in the other two species. The values presented subsequently are not corrected for this fall-off. Increasing the concentration of GA to as much as 75 mM and pyridoxal phosphate to as much as 32 mM did not prevent the fall-off of activity with time. Preincubation of the brain

* When the reaction of ninhydrin with γAB is to be carried out in the absence of TCA, the ninhydrin is prepared in pH 9·4 buffer.

† A blank for a given amount of incubated homogenate is run by adding TCA directly to the same amount of homogenate before incubation. When using frozen-dried tissue exactly the same amount of tissue cannot be obtained as a blank for each incubated tissue sample. For this reason, the blank must be calculated from two readings: (a) that given by pieces of frozen-dried tissue to which TCA is added before incubation, and (b) that given by the buffer-substrate alone to which TCA is added. The blank divisions per μg of tissue are then calculated by subtracting the substrate blank (b) from each tissue blank (a), and dividing the resulting values by the μg of tissue in each tissue blank. These values are then averaged to obtain the mean fluorimeter divisions per μg of unincubated tissue. The net increase in fluorimeter divisions due to the active tissue is its total reading minus [the reading of the substrate blank + (μg active tissue × average blank divisions per μg of tissue)].

§ This fall off of activity with time in homogenates has also been found by Dr. Eugene Roberts (personal communication). He did not find this fall-off when using acetone powders (Roberts and Frankel, 1951). This difference in behaviour of homogenates and acetone powders is presently being investigated.

homogenate indicated that the fall-off with time was not due to enzyme instability. Incubation in the presence of added γAB indicated that the enzyme was not inhibited by this product.

The activity of the enzyme is proportional to brain concentration over a fifteen-fold range, from 2·6 μg/μl to 42 μg/μl (Fig. 2). Even with 84 μg/μl there is only an 8 per cent fall in activity. The total blank for the enzyme measurement is made up of two

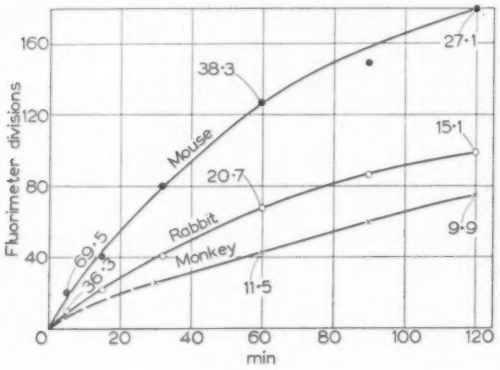

FIG. 1. GDC activity with time in the brains of three species. Brain concentration during enzyme incubation was 50 μg per μl. It was shown that this fall-off was not prevented when the brain concentration was 10 μg per μl. The values indicated by arrows are the enzymic activities in m-moles γAB formed per kg wet brain per hr.

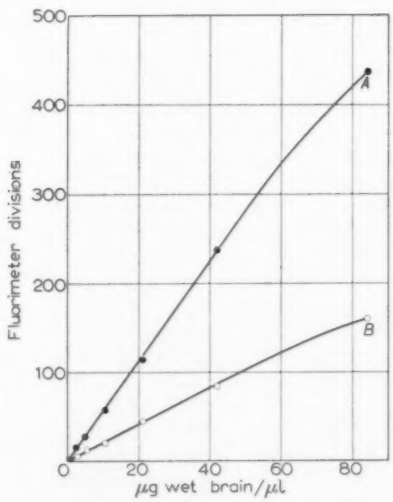

FIG. 2. Relation of enzymic activity and of brain blank to brain concentration. Curve *B* = the brain blank due to fluorescent ninhydrin-reactive, TCA-soluble substances. This curve was obtained by subtracting the fluorescence due to the substrate from the fluorescence of the substrate when mixed with the indicated concentrations of TCA-treated brain. Curve *A* = the fluorescence due to the γAB formed during the enzymic reaction. This curve was obtained by subtracting from the readings of the total brain the readings of the total blank, which are the values in curve *B* plus 23 divisions due to the fluorescence of the substrate.

parts: (a) a substrate blank which results from the fluorescence of the GA-ninhydrin product, and (b) a tissue blank which results from the fluorescence of TCA-soluble, ninhydrin-reactive materials present in brain. The ultimate sensitivity of the method is limited by the substrate blank: otherwise it would be possible to measure the amount of activity in less than 3 μg of brain. In a 1 hr incubation period, the tissue blank limits the maximum ratio that can be obtained between readings for active brain and the total blank to approximately from 2 to 2·5 because, as the concentration of brain incubated is increased, the increase in the brain blank is proportional to the increase in the reading due to active brain (Fig. 2, curves *A* and *B* are proportional throughout). Even though there is this upper limit on the ratio of active brain to total blank, the

precision of the method is satisfactory. With only 3·7 μg of brain, the coefficient of variation is only 5·6 per cent (Table 1).

The recovery of added γAB in four experiments averaged 95 per cent, ranging from 92 to 100 per cent when 2·0 μmoles of γAB were added to 100 mg. dry brain (92, 94 and 95 per cent recovery) and when 1·08 mμmoles were added to 7·1 μg dry brain (100 per cent recovery).

TABLE 1.—REPRODUCIBILITY OF MEASUREMENTS OF GDC IN MOUSE BRAIN

Incubation conditions as described in text. Weight of tissue used was 3·5 μg dry weight. Activities expressed as millimoles of γAB formed per kg dry brain per hr.

Determination no.	Activity
1	118
2	126
3	117
4	126
5	124
6	134
7	126
8	135
9	133
10	134
11	135
12	136
Mean	128
S.D.	7·2
Coefficient of variation (%)	5·6

Comment on ninhydrin reaction. The measurement of GDC requires 25 mM-GA during the enzyme reaction for optimal activity. As a result, during the reaction with ninhydrin approximately 4 mM-GA is present. This is a great excess over the concentration of γAB formed, which approximates 0·1 mM. The reaction of γAB with ninhydrin in the presence of GA differs greatly from the reaction when γAB and ninhydrin are allowed to react in the absence of GA. This difference in the reaction, depending upon whether GA is present or not, is very important in making the measurement of very small concentrations of γAB possible. The reaction in the absence of GA will be described first and the increased sensitivity achieved in the presence of GA will then be described.

The reaction of ninhydrin with γAB in the absence of GA is proportional to the concentration of γAB over a range of only from 1 to 3 mM (Fig. 3). At concentrations of γAB below 1 mM the amount of fluorescence per mole of γAB increases with increasing γAB concentration, suggesting that 1 mole of ninhydrin reacts with more than 1 mole of γAB to form the fluorescent product. The same relationship obtains with GA below 1·5 mM when it is allowed to react alone with ninhydrin. In the reaction with γAB, at 4 mM there is a slight fall from proportionality and above this

concentration a marked fall; with GA, proportionality with concentration obtains up to at least 9 mM.

The reaction of γAB with ninhydrin in the presence of optimal amounts of GA is strikingly different from that when γAB is allowed to react alone (Fig. 4). There are three differences: (a) proportionality with γAB concentration is achieved at a level of 0·01 mM instead of at 1 mM; (b) the range of proportionality is greatly extended,

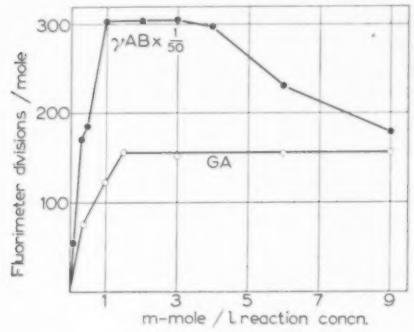

FIG. 3. Relationship between the molar fluorescence produced and the concentrations of γAB and of GA when allowed to react separately with ninhydrin.

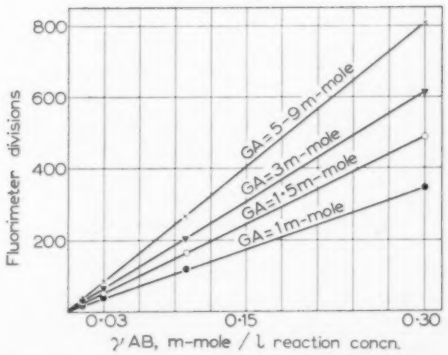

FIG. 4. Relationship between the fluorescence and concentration of γAB when allowed to react with ninhydrin in the presence of optimal amounts of GA. These curves remain linear at least to a γAB concentration of 1 mM.

covering a hundred-fold range (0·01–1·0 mM) instead of only a three-fold range; and, (c) the amount of fluorescence per mole of γAB is approximately a hundred times greater. The optimal levels of GA concentration are from 1 to 9 mM. This range includes the concentrations of GA present during the reaction with ninhydrin when measuring GDC. Between these extremes the reaction of ninhydrin with γAB is linear. Below 1 mM-GA, linearity is not achieved. For example, in the presence of 0·2 mM-GA, the fluorimeter readings per mole of γAB for 0·11, 0·037, and 0·012 mM-γAB are 6·2, 4·2, and 3·4 divisions, respectively. With no GA present, the fluorimeter readings for the same concentrations of γAB are 2·3, 0·7, and 0·4 divisions, respectively. Between 1 and 5 mM-GA, the amount of fluorescence per mole of γAB increases with increasing concentrations of GA, without affecting the proportionality at a given level of GA. Above 5 mM-GA there is no further increase in fluorescence per mole of γAB.

The optimum pH for the reaction of ninhydrin with γAB in the presence of GA is between 9·0 and 10·6. At pH 7·5 there is a 10 per cent decrease in the amount of fluorescence, at pH 11·0 a 15 per cent decrease, and pH 12·5 a 90 per cent decrease.

The optimum pH for reading the fluorescence after dilution with the copper tartrate reagent is between 6·8 and 10·1. In the assay as described the pH after dilution is 9·8–10·0. There is a marked decrease in sensitivity only after reaching pH 4·5 or pH 12. All of these results were obtained in the presence of carbonate buffer, since borate, tris 2-amino-2-hydroxymethyl-propane-1:3-diol, and 2-amino-2-methyl-1:3-propanediol caused a marked inhibition of fluorescence. The concentration of carbonate made a negligible difference in the amount of fluorescence developed.

The optimum concentration of ninhydrin when allowed to react with γAB in the presence of GA is 8 to 10·6 mM. With 2·6 mM-ninhydrin, 50 per cent less fluorescence is obtained; with 13·3 mM, 15 per cent less; and with 26·0 mM, 72 per cent less.

The time required to complete the ninhydrin reaction at 38° is 4 hr, at 50°, 90 min, and at 60°, 20 min. There is no decrease in fluorescence even when heating is prolonged to 6 hr, 3 hr, and 90 min at 38°, 50°, and 60°, respectively. Even though there is 2·2 times as much fluorescence produced at 38° as at 60°, the ratio of the reading to the blank does not improve because the blank reading is also higher at 38°. For convenience, 30 min at 60° was chosen for routine analysis.

In order to have optimal substrate concentration during the enzyme incubation, the concentration of GA in the reaction mixture is approximately forty times the concentration of γAB formed. The presence of GA, therefore, makes a considerable contribution to the blank, which must be greatly reduced in order for the method to be feasible. The addition of Cu^{++} as copper tartrate after the completion of the ninhydrin reaction reduces the blank fluorescence due to the GA–ninhydrin product by 95 per cent, while only causing a 10–15 per cent reduction in the fluorescence of the γAB–ninhydrin product. The reduction in the fluorescence of the blank due to the brain is negligible at this copper concentration. A period of 10 min is required for the copper to exert its maximum quenching effect. For this reason, it is recommended that readings should not be made until 15 min after the addition of the copper tartrate. The optimal concentration of copper is approximately 1·2 mM* (range 1–2 mM). At this concentration it quenches the GA–ninhydrin fluorescence over at least a seven-fold range of GA concentration, 0·1 to 0·7 mM.*

The fluorescence spectra, obtained in an Aminco-Bowman spectro-photofluorimeter of the reaction products of ninhydrin with γAB, GA, γAB and GA together, and the TCA–supernatant fluid from tissue blanks and from active brain, were identical. The maximum excitation occurred at 375 mμ and the maximum fluorescence appeared at 485 mμ.

The reaction of ninhydrin with γAB, GA, and other amino acids and with some indole derivatives was also investigated. The reaction conditions studied were restricted to the pH and buffer optimal for the reaction with γAB, but these studies were made without added GA present and without Cu^{++} being added. Under these conditions γAB gave the maximum fluorescence of any compound tested (Table 2). When compared with γAB and GA, the compounds studied fell into two groups. The fluorescence per mole of γAB and GA and of the twenty-three compounds in group *A* (Table 2) increases with increasing concentration, although γAB and GA become linear at concentrations where the other compounds are still showing a slight increase. The behaviour of the nine compounds in group *B* (Table 2), which

* These concentrations refer to the final concentrations in the fluorimeter tubes after the copper tartrate reagent has been added.

includes all five indole derivatives studied, is markedly different. These compounds with increasing concentration show a decreasing amount of fluorescence per mole. The relationships between the amount of fluorescence produced and the structural formulas of γAB and the compounds in group *A* suggest that, in the majority of instances, the reaction mechanism is similar to that for γAB and depends on structural

TABLE 2.—REACTION OF NINHYDRIN WITH SELECTED AMINO ACIDS AND INDOLE DERIVATIVES

Each compound (20·9 μl of 0·6, 4·5 and 9·0 mM-aqueous solutions) was mixed with 42·0 μl of 14 mM-ninhydrin in pH 9·4, 0·5 M-carbonate buffer and heated at 60° for 30 min. To each tube 1·0 ml of 0·33 M-$NaHCO_3$ was added and the fluorescence measured as described in the text. Values are expressed as arbitrary fluorimeter divisions per mole of each compound.

Compound	Concentrations (mM) of each compound during the reaction		
	0·2	1·5	3·0
γAB	4700	15,100	15,000
GA	400	2010	2030
Group *A*			
Phenylalanine	1950	8000	9300
β-Amino*iso*butyric acid	850	3790	5550
Arginine	320	1490	1930
Leucine	190	820	1850
Norleucine	140	800	1270
Methionine	120	650	1020
α-Aminobutyric acid	110	470	820
β-Alanine	140	330	460
Histidine, tyramine, tyrosine, glutamine	25–125	95–210	150–320
Taurine, aspartic acid, serine, carnosine, alanine, valine, glycine, *iso*leucine, asparagine, α-amino*iso*butyric acid, $(NH_4)_2SO_4$	0–35	1–40	1–60
Group *B*			
Serotonin	7500	3540	2060
Tryptamine	3070	2040	1780
5-Hydroxyindole-3-acetic acid	1620	1130	1000
Proline		930	840
Lysine	730	530	360
5-Hydroxytryptophan	1000	420	
Cysteine	470	300	240
Tryptophan	260	210	
Hydroxyproline	150	35	25

factors such as, for example, the position of the amino group relative to the carboxyl group, the proximity of a carbon branch to the amino group, the length of the carbon chain, and the presence of a benzene ring.

Distribution of GDC in the Nervous System

The activity of GDC was measured in whole brain homogenates of six mice, two rats, six rabbits, and three monkeys. There were marked differences among the

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species. The average values in millimoles of γAB formed per kg wet brain per hr were: mouse, 35·7; rat, 26·4; rabbit, 19·7; and monkey, 10·5.

Quantitative histochemical data were obtained on pieces of frozen-dried tissue from the three layers of the cerebellum of a monkey (Table 3). GDC in the granular layer was approximately 20 per cent greater than in the molecular layer. A striking finding was the virtual absence of activity in the subjacent white matter.

TABLE 3.—QUANTITATIVE HISTOCHEMICAL RESULTS
The frozen-dried sections analysed weighed 3 to 5 μg. Values are expressed as mM of γAB formed per kg dry brain per hr. One animal of each species was studied.

Species and region of brain	Activity ± S.E.M.
Monkey cerebellum	
Molecular layer (7)	50·4 ± 3·5
Granular layer (7)	61·0 ± 2·2
Subjacent white matter (7)	<0·1*
Rabbit	
Optic tract (5)	<0·1*
Optic nerve (5)	<0·1*
Habenulopeduncular tract (5)	6·99 ± 2·2
Rat cerebellum	
Subjacent white matter (6)	<0·1†

Figures in parentheses are the number of determinations.
* Each individual measurement showed no activity.
† Five measurements showed no activity; one was 2·1.

To investigate further this finding of a very low level of activity in monkey white matter, GDC in three white tracts of a rabbit and in the cerebellar white matter of a rat was studied using the quantitative histochemical method (Table 3). In two of the three rabbit white tracts and in the rat cerebellar white matter negligible activities were found. There was an appreciable activity in the habenulopeduncular tract of the rabbit. Even though only 4–5 μg pieces of this tract were used, it was still possible, because of the histological characteristics of this very small tract, that there were bars of grey matter crossing it which could not be avoided. The high standard error of the mean for the values in this tract suggested that there may have been such variation in the histological sampling.

To study further the distribution of this enzyme in the brain, GDC was measured in larger areas of monkey and rabbit brain (Table 4). In all areas examined rabbit brain was more active than the corresponding area of monkey brain. There was a five- to six-fold difference between the most and least active areas of grey matter in the monkey. The white matter areas showed activities varying from a maximum of 10 per cent of that of whole brain to negligible values. In these grossly dissected areas there is always the chance of some contamination of pure white matter by small amounts of grey matter. It may be estimated that activity in white matter, based on dry weight, is from twenty to over five hundred times less than in whole brain, with the likelihood

TABLE 4.—DISTRIBUTION OF GDC IN THE MONKEY AND RABBIT NERVOUS SYSTEMS

These values were measured in triplicate using homogenates (400 μg wet brain) from the indicated areas. The values for the monkey are the mean of measurements on two animals; for the rabbit values, one animal was used. Values expressed as mM of γAB formed per kg wet brain per hr.

Region of brain	Monkey	Rabbit
Whole brain	10·5	19·7
Grey matter		
Globus pallidus	33·4	
Superior colliculus	21·0	36·9
Hypothalamus	20·5	26·7
Visual cortex	18·4	
Cerebral cortex, unspecified		16·2
Putamen	16·4	
Caudate nucleus, head	16·2	21·7
Uncus and amygdaloid nucleus	14·7	
Motor cortex	13·3	
Thalamus	13·2	
Midbrain reticular substance	11·1	
Ammon's horn	9·9	16·6
Cerebellum, whole	9·7	11·8
Lateral geniculate body	6·5*	
Medulla†	4·8*	11·3
Cervical spinal cord†	1·4	2·3
White matter		
Cerebellum	1·0*	
Cerebral peduncle	1·0*	
Cerebral (subcortical)	0·29*	2·8
Medullary pyramid	0·28*	
Internal capsule	0·23*	
Cervical cord, white only	0·13*	0·17
Corpus callosum	0·1	
Brachium pontis	0·1*	
Optic nerve	0·1*	
Dorsal ganglion	0·61*	
Sciatic nerve		0·16

* Values from a single animal.
† These two areas contain appreciable amounts of white matter.

that the white matter with the greatest activity is at least fifty times less active than whole brain.

DISCUSSION

The measurement of GDC by the fluorescence of the γAB formed during the enzymic incubation is advantageous from the standpoints of sensitivity, reproducibility, and convenience. The general reaction of ninhydrin with other amino acids and indole derivatives to form fluorescent products is also useful for other analytical purposes. For example, under optimal conditions for the measurement of phenylalanine, this reaction is being used in this laboratory to measure peptidases

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which split phenylalanine-containing peptides. The reaction with phenylalanine becomes linear at very low concentrations and shows an increased fluorescence (measurable at 5×10^{-8} M) when the reaction is carried out in the presence of excess glycyl-L-phenylalanine. The optimal conditions for each of the other compounds must therefore be determined. The presence of an ancillary compound in excess (GA for γAB and glycyl-L-phenylalanine for phenylalanine) appears to be a means of making these reactions more sensitive and linear at very low concentrations.

Wide differences in the activity of GDC were found in the four species studied. The ratios of activities, taking the monkey as 100, were 187 for the rabbit, 251 for the rat, and 340 for the mouse. When individual areas of rabbit and monkey brain were compared, GDC was found to be richer in the rabbit for each area studied.

The finding that γAB has profound inhibitory effects on transmission in the central nervous system (FLOREY, 1954; BAZEMORE, ELLIOTT and FLOREY, 1956; PURPURA, GIRADO and GRUNDFEST, 1957; EDWARDS and KUFFLER, 1957) suggests that perhaps the distribution of GDC may parallel the concentration of inhibitory synapses in different regions of the nervous system. The cerebellar cortex is thought to have less inhibitory synapses than the cerebral cortex (PURPURA, GIRADO and GRUNDFEST, 1957). The present data show an average GDC activity of 56 m-moles per kg dry brain per hr in the molecular and granular layers of the monkey cerebellar cortex, as compared with 92 in the visual (cerebral) cortex and 66 in the motor (cerebral) cortex. While these findings are in the expected direction, the magnitude of these differences is not particularly striking. This may be because GDC has other functions in the nervous system. Until isolated inhibitory synapses and cells (RENSHAW, 1941) are chemically analysed, the values found for GDC in mixed tissues may be the sum of its unknown functions and its postulated function in generating γAB specifically for its effects on synaptic transmission. It is also possible that there is a balance of GDC and γAB-α-ketoglutaric transaminase (BESSMAN, ROSSEN and LAYNE, 1953; ROBERTS and BREGOFF, 1953) to effect a given level of γAB, so that the interpretation of GDC activity in a specific site would also require a knowledge of the γAB-α-ketoglutaric transaminase level at this same site.

The virtual absence of GDC in white matter is unique among the more than fifteen enzymes previously studied in this laboratory. The nature of this low activity in white matter is being further investigated in order to determine whether it is due to a quantitative difference in the amount of activity of the enzyme or whether there is some inhibitory factor in white matter which prevents the full expression of GDC activity. Preliminary data indicate that if there is an inhibitory factor, it could not explain these very low values in white matter. The finding that white matter is so poor in GDC suggests that each of the components of white matter—axis cylinders, myelin, oligodendroglia, and astrocytes—contains little or none of the enzyme and that GDC is not quantitatively important in their metabolism.

SUMMARY

(1) A sensitive fluorimetric method potentially useful for the measurement of certain amino acids and indole derivatives has been described. It takes advantage of the fluorescent products formed when ninhydrin is allowed to react with these compounds under alkaline conditions. γAB can be measured in a final concentration of 10^{-7} M.

(2) This method has been utilized to determine GDC activity in as little as 3·5 μg dry weight of brain.

(3) Levels of GDC activity were markedly different in the four species studied.

(4) The distribution of GDC in various regions of grey matter in the monkey brain showed a five-fold range of activity, with the globus pallidus exhibiting the highest activity. Quantitative histochemical studies of the monkey cerebellar cortex showed that the granular layer had slightly more activity than the molecular layer, but that the subjacent white matter had a negligible activity.

(5) Other white matter tracts in three species studied had singularly low to negligible amounts of GDC activity.

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THE VALIDATION OF THE QUANTITATIVE HISTOCHEMICAL METHOD FOR USE ON POST MORTEM MATERIAL—II

THE EFFECTS OF FEVER AND URAEMIA*

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THE direct biochemical study of diseases of the central nervous system would be greatly facilitated if it could be shown that brains removed at autopsy could be used for quantitative chemical studies. This question is especially relevant for the study of enzymes, which might be expected to show deterioration or diffusion artifacts beginning a short time after death. The quantitative histochemical method (LOWRY, 1953) has minimized problems of destruction and diffusion of enzymes due to embedding, fixing, and staining, and at the same time has permitted rather precise structural-chemical correlations to be made. This report is concerned with certain aspects of the validation of this technique for use on human autopsy material. The extension to autopsy material is important because human neurological and psychiatric disease cannot, in general, be reproduced in experimental animals, and because sufficient brain tissue cannot ordinarily be obtained by biopsy to study adequately any metabolic disturbances. The special place of the quantitative histochemical methods in studying the biochemistry of such a complex anatomical structure as the brain has been discussed (LOWRY, 1953; ROBINS, 1957; POPE, 1955).

A previous study (SMITH, ROBINS, EYDT, and DAESCH, 1957) of post mortem changes in seven enzymes at 2 and 6 hr after death in three cerebellar layers of normal rabbits showed satisfactory post mortem stability for 6 hr for six of the seven enzymes studied. The present study extends these observations to include the effects of experimentally induced fever or uraemia on two of the previously studied enzymes and on eight additional enzymes. The results of the previous and present investigations show that fourteen of the fifteen enzymes show satisfactory stability, and that fever and uraemia do not affect the enzymes deleteriously during a post-mortem period of 2 and 6 hr.

METHODS

Two types of experiment were performed. In the first, enzymic measurements were made on the cortical layers and the immediately subjacent white matter in the cerebella of uraemic or febrile rabbits after different post mortem periods. In the second, measurements of the same enzymes in water homogenates of brain that were allowed to stand at room temperature for various intervals were made, to see if information concerning the post mortem stability of these enzymes could be obtained by this simpler procedure. The enzymes measured in the febrile animals were aldolase (LOWRY, ROBERTS,

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WU, HIXON and CRAWFORD, 1954), adenosinetriphosphatase (LOWRY *et al.*, 1954), glutamic dehydrogenase (LOWRY, ROBERTS and LEWIS, 1956),* glucose-6-phosphate dehydrogenase (ROBINS, SMITH and JEN, 1957), phosphoglucoisomerase (LOWRY, ROBERTS and CHANG, 1956), and malic dehydrogenase (ROBINS, ROBERTS, EYDT, LOWRY and SMITH, 1956); and, in the uraemic animals, β-glucuronidase (MEAD, SMITH and WILLIAMS, 1955),† fumarase (LOWRY *et al.*, 1954), glutamic dehydrogenase

TABLE 1. TEMPERATURES IN RABBITS MADE FEBRILE BY INJECTION OF TYPHOID VACCINE

A calibrated rectal thermometer was inserted into each rabbit and a baseline temperature measured at 60, 30 and 1 min before the intravenous injection of 1 ml of typhoid vaccine (monovalent reference standard NRV-LS no. 1, made from *S. typhosa* V-58; there were approximately 500,000,000 bacteria per ml). Temperatures were recorded at 30, 60, 90, 120 and 210 min after the injection. The preinjection temperatures are the mean of the three readings taken before injection.

Rabbit no.	Preinjection temperature (°c)	Maximum rise (°c)	Duration of rise >0·5° (hr)
1	37·5	1·1	1·7
2	38·3	1·9	2·3
3	38·3	0·8	1·5
4	37·7	2·4	3·0
5	39·7	1·9	3·0
6	38·8	1·1	1·7

TABLE 2. PLASMA NON-PROTEIN NITROGEN VALUES IN RABBITS MADE URAEMIC BY BILATERAL URETERAL LIGATION

All values are expressed as mg % non-protein nitrogen

Rabbit no.	Operation	Non-protein nitrogen values		
		Prior to operation	24 hr post-operative	48 hr post-operative*
7	Sham†	25	27	33
8	Sham†	30	35	42
9	Ligation	33	60	200
10	Ligation	23	72	170
11	Ligation	23	90	150
12	Ligation	17	75	165
13	Ligation	38	83	145
14	Ligation	23	63	140

* The animals were sacrificed at the time this blood sample was drawn.

† Sham operation = exposure and mobilization of kidneys and ureters bilaterally, but without ligation.

* The absolute values for glutamic dehydrogenase activity reported in this paper are higher than those in the first paper of this series because the enzyme is being measured in the more favourable direction (conversion of α-ketoglutarate to glutamate). In the previous report the conversion of glutamate to α-ketoglutarate was measured.

† The present method for measuring β-glucuronidase is a slight modification of the original method. The modification will be described in detail elsewhere. The substrate used, 4-methylumbelliferone-β-D-glucuronide, was generously given us by Professor R. T. WILLIAMS.

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TABLE 3. INFLUENCE OF TIME AFTER DEATH AT ROOM TEMPERATURE ON ACTIVITIES OF 6 ENZYMES IN FEBRILE RABBITS

All values are expressed as moles of substrate transformed per kg, dry brain, per hr ± S.E.M. Each value is the mean of from four to eight determinations.

Animal no.	Hours post mortem	Cerebellar layer	Aldolase	ATPase	Glutamic dehydrogenase	Glucose-6-phosphate dehydrogenase	Phospho-glucoisomerase	Malic dehydrogenase
1	0	Mol.	8·32 ± 0·26	5·04 ± 0·31	3·05 ± 0·09	1·57 ± 0·03	89·2 ± 5·2	71·4 ± 2·8
		Gran.	5·75 ± 0·17	3·68 ± 0·32	4·02 ± 0·10	1·03 ± 0·04	82·6 ± 12·1	76·6 ± 1·5
		White	2·26 ± 0·09	1·15 ± 0·08	1·39 ± 0·20	1·54 ± 0·09	33·4 ± 2·2	21·6 ± 0·6
2	0	Mol.	7·49 ± 0·38	4·41 ± 0·43	2·71 ± 0·05	1·55 ± 0·05	97·8 ± 2·1	71·3 ± 0·5
		Gran.	4·89 ± 0·14	3·72 ± 0·32	3·54 ± 0·15	1·05 ± 0·02	74·6 ± 5·4	71·3 ± 1·2
		White	2·71 ± 0·13	1·34 ± 0·16	1·20 ± 0·22	1·33 ± 0·07	32·9 ± 2·1	20·3 ± 0·6
3	2	Mol.	9·37 ± 0·19	4·70 ± 0·67	2·68 ± 0·11	1·51 ± 0·10	96·2 ± 1·8	70·2 ± 0·7
		Gran.	5·11 ± 0·25	4·06 ± 0·42	4·01 ± 0·27	1·03 ± 0·13	83·8 ± 7·3	73·0 ± 3·4
		White	2·72 ± 0·33	1·56 ± 0·06	1·50 ± 0·10	1·21 ± 0·09	33·5 ± 3·8	22·0 ± 1·4
4	2	Mol.	9·24 ± 0·19	4·36 ± 0·21	3·30 ± 0·36	1·42 ± 0·03	88·0 ± 3·5	78·3 ± 3·6
		Gran.	4·99 ± 0·34	3·59 ± 0·37	3·77 ± 0·10	1·12 ± 0·05	83·7 ± 7·0	82·0 ± 3·4
		White	2·52 ± 0·13	1·44 ± 0·11	1·27 ± 0·01	1·31 ± 0·13	35·4 ± 1·6	21·0 ± 1·4
5	6	Mol.	7·49 ± 0·60	5·17 ± 0·42	2·85 ± 0·08	1·50 ± 0·03	80·9 ± 5·2	78·0 ± 2·1
		Gran.	4·67 ± 0·26	4·05 ± 0·42	3·54 ± 0·13	0·88 ± 0·04	68·0 ± 5·5	80·9 ± 1·3
		White	2·67 ± 0·16	1·46 ± 0·13	1·49 ± 0·14	1·03 ± 0·12	31·7 ± 2·5	22·2 ± 1·3
6	6	Mol.	8·28 ± 1·31	4·31 ± 0·46	2·76 ± 0·08	1·34 ± 0·05	85·7 ± 3·5	72·8 ± 2·0
		Gran.	5·03 ± 0·87	3·56 ± 0·30	3·60 ± 0·18	1·05 ± 0·13	82·0 ± 6·0	73·5 ± 2·2
		White	3·27 ± 0·17	1·15 ± 0·16	1·75 ± 0·07	1·48 ± 0·03	37·2 ± 1·9	21·2 ± 0·6

(LOWRY *et al.*, 1956), purine nucleoside phosphorylase (ROBINS, SMITH and MCCAMAN, 1953), and glutamic-oxalacetic transaminase (LOWRY *et al.*, 1956).

Methods for obtaining and weighing the frozen-dried pieces of tissue from the cerebellum have been described or referred to in the first paper of this series (SMITH *et al.* 1957). The pieces of tissue analysed weighed from 0·1 to 3·0 μg, depending on the enzyme to be measured. In the experiment with the water homogenates, from 3 to 25 μg of brain, wet weight, were used.

TABLE 4. MEAN PERCENTAGE CHANGES IN POST MORTEM PERIODS COMPARED TO RESULTS AT ZERO-HOURS IN FEBRILE RABBITS

These values were calculated by averaging the activities of each enzyme in each layer at each post mortem time (see Table 3) and setting the activity at zero-time = 100 per cent.

Cerebellar layer	Aldolase (%)		Adenosinetriphosphatase (%)		Glutamic dehydrogenase (%)		Glucose-6-phosphate dehydrogenase (%)		Phosphoglucoisomerase (%)		Malic dehydrogenase (%)	
	2 hr	6 hr	2 hr	6 hr	2 hr	6 hr	2 hr	6 hr	2 hr	6 hr	2 hr	6 hr
Molecular	+17	−7	−4	0	+4	−2	−6	−9	−1	−11	+4	+5
Granular	−5	−9	+3	+3	+3	−5	+4	−7	+6	−5	+5	+4
White	+5	+19	+21	+6	+7	+24	−13	−13	+4	+4	+2	+3

Febrile animals. Fever was produced in six male albino rabbits weighing from 2·2 to 2·6 kg by injection with typhoid vaccine.‡ The maximal elevation of temperature ranged from 0·8° to 2·4° and the minimal duration of fever of greater than 0·5° was from 90 min to 3 hr in individual animals (Table 1). The animals were sacrificed by air embolism 3·5 hr after the typhoid vaccine was given. At this time the rise in temperature was maximal for five of the rabbits and only 0·1° below the maximum in the other one. The cerebella were removed from one pair of animals immediately and frozen in liquid nitrogen (0 hr post mortem). The intact bodies of the other animals were allowed to remain at room temperature (22°) and the cerebella of the second pair were similarly removed and frozen after 2 hr (2 hr post mortem), and from the third pair after 6 hr (6 hr post mortem). During the 2 hr post mortem interval the body temperatures (rectal) of the four animals decreased an average of 1·5° (range, from 0·9° to 2·6°); after 6 hr, the two remaining animals showed total decreases of 7·6° and 5·3°.

Uraemic animals. Uraemia was produced in six male albino rabbits weighing from 2·3 to 3·8 kg by bilateral ureteral ligation just distal to the renal pelvis.§ To study the possible effects of the operative procedure, two other animals had a sham operation. Plasma non-protein-nitrogen values were measured‖ 3 days prior to the operation and at 24 and 48 hr post-operatively at which time the animals were sacrificed by air embolism (Table 2). The bodies and cerebella were handled in the same way as for the febrile animals, except that the cerebella of the two sham-operated animals were placed into liquid nitrogen immediately after sacrifice. The room temperature in this set of experiments was 27·5°. The body temperatures of the 2 hr animals fell by 2·5° and 2·2°, and for the 6 hr animals by 4·9° and 7·3°.

Brain homogenates. Three additional normal rabbits were sacrificed by cardiac puncture and a 1:5 water homogenate of the cerebellum of each was prepared. A portion of this homogenate was frozen immediately in liquid nitrogen and other portions were allowed to remain at room temperature (24°) for 2, 6, 24 and 48 hr before being similarly frozen. Simultaneous measurements in triplicate were then made on each of these homogenates for each of the enzymes. Bacterial contamination was not excluded.

‡ We wish to thank Dr. ELISHA ATKINS for his assistance with this part of the study.
§ We wish to thank Dr. HAROLD PAXTON for his assistance with the operative procedure.
‖ These were measured in the clinical chemistry laboratory of Barnes Hospital.

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RESULTS

Fever. The results of the experiment on the effects of fever on six enzymes immediately post mortem and after 2 and 6 hr post mortem are summarized in Tables 3 and 4. The enzymes taken as a whole showed deviations from the zero-time values of only ±6·6 per cent. The greatest difference from the control was a single value of +24 per cent. Comparisons of the 6 hr values with 2 hr values indicate no progressive or marked change in any of the three layers for any individual enzyme. Comparisons of the results for any individual enzyme in adjacent layers give no clear evidence of

TABLE 5. INFLUENCE OF TIME AFTER DEATH AT ROOM TEMPERATURE ON ACTIVITIES OF FIVE ENZYMES IN URAEMIC RABBITS

All values are expressed as moles of substrate transformed per kg, dry brain, per hr ± S.E.M Each value is the mean of from four to eight determinations.

Animal no.	Hours post mortem	Cerebellar layer	Fumarase	Glutamic dehydrogenase	Glutamic-oxalacetic transaminase	Purine nucleoside phosphorylase	β-glucuronidase†
7*	0	Mol.	24·0 ± 0·8	2·70 ± 0·04	42·9 ± 1·6	0·659 ± 0·018	16·8 ± 1·1
		Gran.	25·3 ± 0·8	3·65 ± 0·07	39·2 ± 2·1	0·676 ± 0·043	17·5 ± 1·0
		White	3·74 ± 0·39	1·50 ± 0·06	15·4 ± 0·5	0·450 ± 0·024	7·24 ± 0·71
8*	0	Mol.	29·7 ± 0·9	2·75 ± 0·05	41·3 ± 0·9	0·479 ± 0·073	13·9 ± 1·3
		Gran.	29·6 ± 1·2	3·77 ± 0·14	40·4 ± 1·5	0·580 ± 0·038	14·5 ± 0·5
		White	3·91 ± 0·63	1·22 ± 0·05	12·6 ± 0·5	0·287 ± 0·033	6·01 ± 0·39
9	0	Mol.	29·1 ± 0·9	2·71 ± 0·05	46·0 ± 1·4	0·463 ± 0·046	13·5 ± 0·6
		Gran.	28·9 ± 1·9	3·70 ± 0·14	42·5 ± 0·7	0·534 ± 0·014	13·7 ± 0·5
		White	3·63 ± 0·10	1·44 ± 0·10	13·9 ± 0·8	0·285 ± 0·045	7·19 ± 0·03
10	0	Mol.	34·2 ± 0·7	3·25 ± 0·07	43·3 ± 1·3	0·648 ± 0·017	13·0 ± 0·8
		Gran.	35·1 ± 0·8	3·53 ± 0·12	43.0 ± 1·8	0·605 ± 0·045	14·3 ± 0·6
		White	3·98 ± 0·50	1·26 ± 0·15	11·6 ± 0·8	0·304 ± 0·041	5·62 ± 0·38
11	2	Mol.	32·5 ± 0·5	2·72 ± 0·07	37·9 ± 0·5	0·480 ± 0·036	12·4 ± 1·1
		Gran.	31·3 ± 0·6	3·66 ± 0·03	34·7 ± 1·6	0·517 ± 0·063	14·6 ± 0·7
		White	3·98 ± 0·33	1·21 ± 0·07	14·4 ± 0·7	0·360 ± 0·039	7·28 ± 0·96
12	2	Mol.	28·9 ± 2·0	3·22 ± 0·04	40·2 ± 1·2	0·556 ± 0·028	11·8 ± 0·8
		Gran.	26·3 ± 0·5	3·67 ± 0·08	37·8 ± 1·4	0·578 ± 0·059	14·0 ± 0·3
		White	3·38 ± 0·24	1·35 ± 0·16	12·8 ± 0·4	0·330 ± 0·018	5·63 ± 0·39
13	6	Mol.	33·0 ± 1·5	3·12 ± 0·19	42·3 ± 2·0	0·524 ± 0·009	10·0 ± 0·7
		Gran.	31·8 ± 2·9	3·81 ± 0·07	38·0 ± 0·9	0·547 ± 0·042	12·8 ± 0·6
		White	3·77 ± 0·33	1·04 ± 0·05	12·1 ± 1·2	0·314 ± 0·022	4·87 ± 0·32
14	6	Mol.	33·0 ± 1·0	2·66 ± 0·08	37·9 ± 0·9	0·507 ± 0·008	16·9 ± 0·7
		Gran.	31·2 ± 2·1	3·23 ± 0·08	37·8 ± 1·0	0·540 ± 0·040	17·6 ± 1·4
		White	3·28 ± 0·28	0·83 ± 0·06	11·0 ± 0·2	0·242 ± 0·007	7·63 ± 1·22

* These animals were sham-operated.
† Units are millimoles of substrate transformed per kg dry brain per hr.

diffusion or migration of the enzyme, even though samples from adjacent layers were often only 50 μ apart and never more than 300 μ.

The data of these experiments were analysed statistically by analysis of variance (OSTLE, 1954). Of the eighteen possible statistical comparisons (three layers for each of six enzymes), there were no significant differences among the results for the various post mortem intervals in sixteen of them. The only significant differences (at the 1 per cent level) were for aldolase in white matter and for phosphoglucoisomerase in the molecular layer. The change in aldolase activity was only +19 per cent at 6 hr and in phosphoglucoisomerase activity only —11 per cent. These results are presumably due to histological differences between animals or differences in the locus in the cerebellum

TABLE 6. MEAN PERCENTAGE CHANGES IN POST-MORTEM PERIODS COMPARED TO RESULTS AT ZERO-HOURS IN URAEMIC RABBITS

These values were calculated by averaging the activities of each enzyme in each layer at each post mortem time (see Table 5) and setting the activity at zero-time = 100 per cent.

Cerebellar layer	β-glucuronidase (%)		Fumarase (%)		Glutamic dehydrogenase (%)		Purine nucleoside phosphorylase (%)		Glutamic oxalacetic transaminase (%)	
	2 hr	6 hr	2 hr	6 hr	2 hr	6 hr	2 hr	6 hr	2 hr	6 hr
Molecular	−9	+1	−3	+4	0	−3	−7	−7	−12	−10
Granular	+2	+8	−10	−2	+1	−3	−4	−5	−15	−11
White	+1	−2	−3	−7	−5	−32	+17	−6	+6	−9

chosen for sampling, since there was no evidence in the other layers of such a marked change with lapse of time post mortem for either of these enzymes.

Uraemia. The results of the experiment on the effects of acute uraemia on the five enzymes immediately post mortem and after 2 and 6 hr post mortem are summarized in Tables 5 and 6 (the sham-operated animals will be discussed below). The enzymes taken as a whole showed deviations from the zero-time values of only $\pm$6·8 per cent. The greatest difference from the control was a single value of —15 per cent, except for glutamic dehydrogenase in white matter at 6 hr, which showed a —32 per cent change. Comparisons of the 6 hr values with the 2 hr values indicate no consistent and marked change for any of the enzymes except glutamic dehydrogenase. This enzyme showed a marked change in only one of the three layers, the white matter. Comparisons of the activities of any individual enzyme in adjacent layers give no evidence of diffusion or migration of the enzyme.

Of the fifteen possible statistical comparisons (analysis of variance), there were no significant differences among the results for the various post mortem intervals in twelve of these comparisons.* There was a significant difference (at the 1 per cent level) for glutamic-oxalacetic transaminase in the molecular and granular layers. These differences were, however, only —10 and —11 per cent, respectively, at the end of 6 hr. These values were actually a slight increase from those at 2 hr. This indicates

* It is possible that significant differences due to lapse of time after death could have been obscured for β-glucuronidase in all three layers and for purine nucleoside phosphorylase in the molecular layer. The reason for this is that a statistically significant variance was contributed by differences between some rabbits within the same post-mortem interval for these enzymes in these locations. Inspection of the results (Table 5) indicates, however, that no serious and progressive deterioration occurred.

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that the enzyme was not undergoing any serious and progressive deterioration even 6 hr after death. The decrease of −32 per cent in glutamic dehydrogenase activity in white matter, on the other hand, does indicate a very marked fall in the activity of this enzyme. The findings that this enzyme did not change significantly in normal animals (SMITH *et al.*, 1957), in the febrile animals, and in two of the three cerebellar layers in the uraemic animals raises the question whether there is a specific effect of uraemia on the post mortem stability of this enzyme in white matter, or whether this is a unique occurrence and would not occur again on replication of these experiments.

The zero-hour uraemic animals showed differences from the sham-operated animals which averaged only ±7·8 per cent (Table 5). Fumarase activities in the molecular and granular layers showed the greatest differences. There was an 18 and 16 per cent increase, respectively, in the uraemic animals when compared with the sham-operated animals.

Brain homogenates (Table 7). All of the ten enzymes studied showed a relatively

TABLE 7. EFFECT OF STANDING AT ROOM TEMPERATURE ON THE ENZYMIC ACTIVITIES OF WATER HOMOGENATES OF CEREBELLUM FROM THREE RABBITS

Values are expressed as per cent of the zero-time value, which was taken as 100 per cent

Enzyme	Mean (M) and range (R)	Time at room temperature (24°) (hr)			
		2	6	24	48
Fumarase	M	108	111	102	
	R	103–117	106–115	99–105	
Glutamic-oxalacetic transaminase	M	98	91	83	65
	R	94–104	89–94	81–85	53–73
Purine nucleoside phosphorylase	M	102	103	103	95
	R	97–107	100–106	98–109	81–103
β-Glucuronidase	M	101	104	99	90
	R	97–104	100–107	91–109	78–103
Aldolase	M	104	106	95	92
	R	100–111	102–111	88–102	84–100
Phosphoglucoisomerase	M	107	108	122	118
	R	99–118	101–115	110–138	101–135
Glucose-6-phosphate dehydrogenase	M	105	92	58	30
	R	89–122	77–114	48–72	23–39
Adenosinetriphosphatase	M	108	112	119	72
	R	99–122	94–138	101–144	58–80
Malic dehydrogenase	M	106	102	101	103
	R	102–108	99–107	98–105	102–106
Glutamic dehydrogenase	M	100	98	87	
	R	99–102	97–99	86–89	

slight change at 2 and 6 hr (±11 per cent or less) and six of the ten showed an insignificant change at 24 hr (±13 per cent). There were four enzymes that showed a change of greater than 15 per cent at 24 hr. Glutamic-oxalacetic transaminase and glucose-6-phosphate dehydrogenase decreased 17 per cent and 42 per cent, respectively. Phosphoglucoisomerase and adenosinetriphosphatase increased 22 per cent and 10 per cent, respectively. These changes at 24 hr in these four enzymes were not reflected in the 6 hr frozen-dried material nor in the 6 hr homogenates. By 48 hr all enzymes except phosphoglucoisomerase and malic dehydrogenase had lost activity.

DISCUSSION

These experiments combined with those reported previously (SMITH *et al.*, 1957) demonstrate the practicability of applying quantitative histochemical methods, as well as other quantitative measurements, for fourteen of the fifteen enzymes so far tested in the central nervous tissue of rabbits after post mortem intervals of at least 6 hr. It it probable that these results would be equally applicable to other mammalian species, including man. As the time intervals are comparable to those that can be obtained in human autopsies, investigations of human central nervous tissues obtained at autopsy are practicable. It must be emphasized that these results were obtained on brain and should not be applied to other organs, such as liver and kidney, without first validating them. Additionally, the previously reported results for phosphofructokinase (SMITH *et al.*, 1957), which showed a 17–44 per cent (depending on the layer) deterioration at 2 hr post mortem and a 60–70 per cent deterioration at 6 hr, indicates the necessity for testing each new enzyme in brain before measuring it on autopsy material.

The results for the fifteen enzymes reported here and in the previous study show a 1 : 1 correlation between the quantitative histochemical results and the results on the homogenates (SMITH *et al.*, 1957, Table 3; Table 7, this paper). There were fourteen enzymes stable for at least 6 hr in both tests. There was one enzyme, phosphofructokinase, unstable in both tests. The explanation for the results on phosphofructokinase may be its known sensitivity to even a slightly acid medium (UTTER, 1947; BUELL, LOWRY, ROBERTS, KAPPHAHN, and CHANG, in press), which presumably develops post mortem and also occurs in glass-distilled water homogenates of brain. The immediate destruction of phosphofructokinase in homogenates can be prevented by homogenization in an alkaline environment. This explanation suggests that the homogenate procedure might not be generally valid as a screening procedure for the quantitative histochemical method. On the other hand, until a definite example of a disagreement between the homogenate and quantitative histochemical procedures is forthcoming, it seems valuable to continue the homogenate procedure as a rapid screening device.

In the first report (SMITH *et al.*, 1957), in which only normal rabbits were studied, the question was raised whether or not certain agonal conditions frequently associated with or leading to death in humans would invalidate these results. In this study it was shown that fever or uraemia did not affect the results for the six enzymes tested in febrile animals or for the five tested in uraemic animals. It is true in both instances that the conditions were relatively acute, 2–3 hr of fever and 2 days of uraemia,*

* The finding that the differences between the sham-operated and zero-hour uraemic rabbits were small, suggests that these enzymes are not involved in the pathogenesis of the neurological and psychiatric symptoms which occur in uraemia. The fact that the uraemia was not chronic, however, urges caution in the interpretation of these findings.

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and that the animals did not die because of the fever or uraemia. In spite of this, it is at least suggestive that such agonal conditions will not seriously affect the results when applied to autopsy material.

Reasons why changes of from 10 to 20 per cent in the activity of a particular enzyme during a post-mortem period of 6 hr were considered insignificant, and reasons why the cerebellum was chosen for study, have been discussed (SMITH *et al.*, 1957).

The fourteen stable enzymes that have been found permits the sampling of a number of different aspects of metabolism in the human brain obtained at autopsy. Among the fourteen enzymes there is at least one enzyme involved in each of the following metabolic transformations: the glycolytic cycle, the tricarboxylic acid (Krebs) cycle, glutamate metabolism, purine metabolism, the hexose 'shunt', overall energy metabolism (adenosinetriphosphatase), hydrolysis of phosphate bonds, primary phosphorylation of glucose (hexokinase), and hydrolysis of glucuronides.

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SUMMARY

Changes in enzymic activities in three layers of the cerebellum of the rabbits as measured by quantitative histochemical analyses, were studied at post mortem intervals of 2 and 6 hr. Five enzymes—fumarase, β-glucuronidase, glutamic dehydrogenase, purine nucleoside phosphorylase, and glutamic-oxalacetic transaminase—were studied in animals made uraemic prior to death. Six enzymes—aldolase, adenosinetriphosphatase, glutamic dehydrogenase, glucose-6-phosphate dehydrogenase, phosphoglucoisomerase, and malic dehydrogenase—were studied in animals made febrile. All eleven enzymes showed a sufficient degree of stability and absence of diffusion artifact for 6 hr post mortem to indicate that valid quantitative results may be obtained for these enzymes in material collected within the usual post mortem interval of human autopsies.

The stability of these enzymes in water homogenates was shown to correlate with the results of the more detailed analyses on frozen-dried material and it was suggested that the homogenate technique may be a simple and rapid means to determine the post mortem stability of an enzyme. The generality of the homogenate screening method is not established, but it appears to be useful as a guide to more detailed experiments.

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OCCLUSION AND METABOLISM OF γ-AMINOBUTYRIC ACID BY BRAIN TISSUE*

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INHIBITION of impulse generation in stretch receptor neutrons of the crayfish by mammalian brain and spinal cord extracts was first described by FLOREY (1954) who called the agent or agents responsible 'Factor I'. A method for assaying Factor I activity was developed by ELLIOTT and FLOREY (1956) and, with the aid of this method, BAZEMORE, ELLIOTT and FLOREY (1956, 1957) isolated from beef brain a strongly active substance which was identified as γ-aminobutyric acid (GABA). Extracts containing Factor I have been found to exert various effects on nervous structures in marine organisms and in mammals (FLOREY, 1956; FLOREY and MCLENNAN, 1955*a*, *b*). HAYASHI and NAGAI (1956) have reported that GABA applied to the cerebral cortex or injected into the carotid artery or cerebrospinal fluid inhibits electrically or chemically induced convulsions in the dog; the β-hydroxy derivative was also active. γ-Aminobutyric acid applied topically to the exposed cerebral cortex has been found to modify profoundly the electrically observed activity of the cerebral cortex of the cat (PURPURA, GIRADO and GRUNDFEST, 1957*a*,*b*,; IWAMA and JASPER, 1957). These observations, and those of KILLAM and BAIN (1957) and KILLAM (1957) on the effects of hydrazide intoxication, all suggest that γ-aminobutyric acid is concerned in the regulation of physiological activity in the brain and other nervous structures.

If a substance is normally involved in such regulation it must be presumed that it can be produced by the tissue, that it can be stored in some inactive condition from which it can be released in the active form when its activity is called for, and that the free active substance can be disposed of when its activity is no longer required. The present report describes studies *in vitro* related to these presumptions.

METHODS

Slices of rat cerebral cortex about 0·5 mm thick were prepared, without moistening, by means of a Stadie–Riggs microtome in a humid chamber. They were weighed and immersed in glucose-containing bicarbonate-buffered saline medium†, 2·5 ml unless otherwise stated, usually in Warburg vessels. Usually eight vessels were set up each containing 100–200 mg of slices sampled from the brains of four rats. The vessels were filled with 95% oxygen–5% carbon dioxide and shaken in a bath at 38°. In anaerobic experiments 95% nitrogen–5% carbon dioxide was used. Added substances, neutralized when necessary, were made up to the required concentration in the usual medium.

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† The medium contained (m-moles/l.): NaCl 122, KCl 3·1, $MgSO_4$ 1·2, $CaCl_2$ 1·3, KH_2PO_4 0·4, $NaHCO_3$ 24·5, glucose 10.

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Brain suspensions consisted of tissue dispersed in a saline medium by means of the Potter–Elvehjem homogenizer. Procedures followed with these suspensions are described in the text and tables below.

Preparation of extracts for assay. At the end of the period of incubation, in experiments with slices, the contents of each Warburg vessel were poured on to a perforated disk in a funnel, very light suction being applied. The slices were usually rinsed with 1 ml of fresh incubation medium (containing no added substance) and placed in a tube with nineteen times their volume of 'unbuffered crayfish saline'* containing only three-fourths the usual concentration of potassium, heated in a boiling water-bath for 15 min and homogenized. When the Factor I content was expected to be low, nine volumes of saline containing half the usual potassium were used. (The potassium present in the incubated slices brought the concentration of potassium in the suspensions up to that of complete crayfish saline.)

The medium from the Warburg flasks, which was collected in a test tube under the funnel when the slices were separated, was heated in a boiling water-bath; 0·1 ml of 'adjustment solution'† and 0·1 ml of 0·11 M-tris-maleate buffer solution were added per millilitre of medium and the pH was adjusted to 6·5 with N-HCl. The addition of adjustment solution brought the electrolyte content of the medium to about the same as that of the crayfish saline. The volume of the medium obtained after filtering off the slices, and its volume after heating and adjustment, were noted for later calculations of the activity in the medium. Further dilutions of the homogenized slices and of the adjusted media for assay were made with buffered crayfish saline.

In experiments with brain suspensions the residue after centrifugation was taken up with buffered crayfish saline, heated and diluted further as necessary. The supernatant fluid was adjusted when necessary in the same way as the incubation medium.

Assay. The assay procedure using the crayfish stretch receptor was similar to that described by ELLIOTT and FLOREY (1956). Solutions of GABA took the place of a brain extract as reference standard. The dissection was considerably simplified for most of the assays.‡ Instead of freeing the nerve from all muscle except the stretch receptor strand, two thin bands of muscle which overlie the nerve, together with the branches of the nerve which supply them, were left uncut; the electrode was connected to the nerve where it emerges near the edge of the exoskeleton. No difference in sensitivity was noted when the sensitivity to GABA was tested before and after doing the complete dissection.

It was previously reported (ELLIOTT and FLOREY, 1956) that, among a number of substances tested, 7 mM glutamic acid (1 mg/ml) exerted no effect on the stretch receptor. In the present study we have found that the effects of glutamic acid are variable. With some receptor preparations no effect was observed while, with some, concentrations as low as 1 mM (150 μg/ml) caused excitation often followed by a block which was only slowly reversible on washing. When solutions containing 5 mM-L-glutamate were first applied, contraction of the muscle was observed, often strong

* 'Unbuffered crayfish saline' contained (m-moles/l.): NaCl 205, KCl 5·4, $MgCl_2$ 2·6, $CaCl_2$ 13·3. 'Buffered crayfish saline' was a mixture of ten parts of the unbuffered solution with one part of 0·11 M-tris-maleate buffer, final pH 6·5, with 0·7 mg/100 ml of bromcresol purple added. As noted below in the text, 5 mM L-glutamate was added to the buffer solution in most cases.

† This 'adjustment solution' contained (m-moles/l.): NaCl 785, KCl 25·5, $CaCl_2$ 136, $MgCl_2$ 15.

‡ This simplification was suggested by Dr. MAX BEN of Merck, Sharp & Dohme Research Laboratories.

enough to cause movement of the exoskeleton. On two occasions low concentrations of glutamic acid, 60 and 20 μg/ml, caused a readily reversible block similar to that obtained with GABA. Sensitivity of the stretch receptor preparation to glutamate decreased and finally disappeared after repeated applications.

Since brain contains considerable amounts of glutamic acid, and in some experiments glutamate was added to the incubation medium, it was necessary to make sure that glutamate did not interfere with the assay of GABA. In a number of experiments the stretch receptor preparations were tested for sensitivity to glutamate and preparations were used only if they were insensitive to higher concentrations of glutamate than were likely to be present in extracts diluted to contain the minimum blocking concentration of GABA.

In later experiments, 5 mM-L-glutamate was added to the buffered crayfish-saline medium which was used for washing the preparation and diluting the GABA standards and extracts. All solutions thus contained a concentration of glutamate which was high compared with the concentration of glutamate, from the tissue or from the incubation medium, which could be present in the extracts as finally diluted for assay. With this glutamate-saline the sensitivity of stretch receptor preparations to GABA seemed to be improved, the minimum blocking concentration being usually 2–5 μg/ml (compared to 3·5–12 μg/ml without glutamate*) and no obvious disadvantages appeared except that the solutions were more liable to infection unless they were stored in the frozen state.

The results of all Factor I assays are expressed in terms of the amounts of GABA which would show the equivalent activity. The assumption that GABA is the only substance in the extracts which affects the stretch receptor neuron has not been proved but it is probably approximately valid in the present work. The disadvantage of this uncertainty is counterbalanced by the advantage of studying changes in actual biological activity. Duplicate assays on individual extracts usually agreed within 10 per cent. Figures given are the mean of at least two assays. Results obtained in replicate experiments with different samples of tissue from the same animals usually agreed within 10 per cent though occasionally greater variations occurred. Examples of replicate experiments are shown in Figs. 3 and 4.

RESULTS

Apparent non-destruction of GABA. No sign of rapid destruction of GABA by brain tissue suspensions or slices has been obtained. ELLIOTT and FLOREY (1956) reported that the total Factor I activity found in heated brain suspensions tended to increase, rather than to diminish, if the suspensions were kept at room temperature for 1 hr before they were heated. Tables 1 and 2 show the results of experiments in which heated brain extracts containing Factor I activity, or known amounts of GABA, were incubated with unheated brain suspensions. There was no evidence of rapid destruction of Factor I activity.† Results given in Table 3, and many others, showed that there is no obvious decrease in the total Factor I activity when slices of cerebral cortex are incubated in the presence or absence of added GABA.

LISSÁK and ENDRÖCZI (1956) reported the presence of an inhibitory factor, prepared

* ELLIOTT and FLOREY (1956) found the minimum blocking concentration without glutamate to be usually about 2 μg/ml, but it varied widely.

† It might be noted that rat cerebral cortex can destroy acetylcholine at a rate of nearly 25 μmoles/100 mg of tissue per hour (see e.g. TOWER and ELLIOTT, 1952).

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TABLE 1.—RECOVERY OF FACTOR I IN BRAIN EXTRACT AFTER TREATMENT WITH BRAIN SUSPENSION

	Extract	Suspension	Extract plus suspension	Recovery* (%)
Unincubated	3·5	1·35	4·9	100
Incubated 2·5 hr at 24°		1·5	5·2	108
Incubated 1 hr at 38°			5·2	108
Unincubated	3·2	0·55	3·85	103
Incubated 2·5 hr at 24°		0·61	3·75	100
Incubated 1 hr at 38°			4·0	107

The 'extract' was prepared as follows: Rat cerebral hemispheres were dropped, in pieces, into K-free unbuffered crayfish saline, heated in a boiling-water bath for 10 min, and homogenized. The homogenate was made to 200 mg tissue/ml with the same saline and centrifuged at 18,000 rev/min. The pH of the supernatant was adjusted to 7·5.

The brain 'suspension' consisted of an unheated homogenate of cerebral hemispheres, 200 mg/ml in the same saline.

Tubes were set up containing 4 ml of extract and 1·2 ml of suspension, or with either of these replaced by crayfish saline. Thus each tube contained extract from 800 mg of brain or suspension of 240 mg of brain or both. Either immediately or after incubation the tubes were heated in a boiling water bath and their contents re-homogenized and assayed.

The figures show the total Factor I activity as μmoles of GABA, in each tube.

* Taking the sum of the separately estimated activities of the extract and the unincubated suspension as 100 per cent.

TABLE 2.—RECOVERY OF ADDED γ-AMINOBUTYRIC ACID (ESTIMATED AS FACTOR I) FROM BRAIN SUSPENSIONS

	Unincubated			Incubated 1 hr at 38°		
GABA added (μmoles/ml)	—	2·0	10·0	—	2·0	10·0
	100 mg tissue/ml					
Total activity found per ml	0·41	2·4		0·41	2·5	
Recovery (%)		100		100	104	
	150 mg tissue/ml					
Total activity found per ml	0·59	2·5	11·2	0·57	2·3	12·7
Recovery (%)		97	106	98	89	120
	300 mg tissue/ml					
Total activity found per ml	1·2	3·2		1·0	4·9	
Recovery (%)		100		83	153	

Factor I activity as μmoles of GABA. The sum of the activity of the unincubated tissue and the GABA added is taken as 100 per cent.

Rat cerebral hemispheres were homogenized in Ringer–phosphate solution. Small Erlenmeyer flasks received suspension and GABA to give the final concentrations indicated in a total volume of 3 ml. The vessels were heated, either at once or after being shaken in air for 1 hr at 38° in an Aminco–Dubnoff apparatus. The suspension was rehomogenized and assayed.

TABLE 3.—DISTRIBUTION OF γ-AMINOBUTYRIC ACID BETWEEN SLICES AND MEDIUM AFTER 60 MIN INCUBATION IN THE PRESENCE OF OXYGEN AND GLUCOSE

Tissue (mg)	GABA in medium (μmoles/ml)		GABA in tissue (μmoles/g*)		Total GABA recovered (%)
	Added	Incubated	Initial	Incubated	
Cerebral cortex slices					
114	0·0	<0.02	3·0	3·3	110
135	2·0	1·3		14·3	96
134	5·0	3·7		20·0	93
400	0	0·01	4·0	4·5	114
682	0·3	0·03		5·2	104
585	2·0	0·15		11·4	96
Diaphragm sectors					
188	0·0	<0·01		<0·1	
172	2·0	1·8		1·9	93
183	2·0	1·9		1·4	100
Liver slices					
256	0·0	<0·02		<0·15	
274	2·0	1·8		0·7	94
254	2·0	1·6		1·2	86
258	2·0	1·8		1·7	99
Kidney cortex slices					
148	0·0	<0·01		<0·1	
182	2·0	1·3		1·0	69
181	2·0	1·8		1·0	94

Representative slices of brain cortex were assayed, without incubation, to give the figure for 'GABA in tissue: Initial'. (Initial figures for other tissues were not obtained in these experiments because these tissues normally contain no detectable Factor I activity and their 'Initial' contents are taken as zero.) Other slices were weighed (weight given under 'Tissue (mg)') and incubated aerobically for 60 min at 38° in 2·5 ml glucose-containing bicarbonate-buffered saline medium containing the concentrations of added GABA shown under 'GABA in medium: Added'. After the incubation period the media and tissue were separately assayed to give 'GABA in medium: Incubated' and 'GABA in tissue: Incubated'.

When no activity was detectable the minimum concentration which would have been detected at the least dilution which could be tested is indicated by the < sign.

The column 'Total GABA recovered' gives the ratio (× 100):

$$\frac{\text{'GABA in tissue: Incubated'} \times \text{wt (g)} + \text{'GABA in medium: Incubated'} \times 2{\cdot}5}{\text{'GABA in tissue: Initial'} \times \text{wt (g)} + \text{'GABA in medium: Added'} \times 2{\cdot}5}$$

* Results are given in terms of the initial weight of slices. Swelling by the tissue would affect the final volume of the medium only slightly and is disregarded.

from brain extracts by ethanol extraction and further purification, which was unstable. We have found no sign of a labile factor which affects the crayfish stretch receptor. Brain was extracted with ice-cold 30 or 70% ethanol, the extract was evaporated under reduced pressure (bath temperature below 18°) and the residue was taken up with buffered crayfish saline the potassium content of which was adjusted to allow for that in the extract. The activity obtained was no greater than that obtained by homogenizing and heating the tissue in saline medium. No activity was lost by the ethanol-extracted material on heating it.

Absorption of GABA by slices. When slices of cerebral cortex were incubated aerobically in glucose-containing saline medium and the Factor I content of the slices and

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medium were then separately assayed, the concentration of Factor I found in the medium was usually below the sensitivity of the assay unless a large amount of tissue had been present in a small volume of medium. The activity was almost all found in the slices and the total amount found was about the same as in unincubated slices.

When known amounts of GABA were added to the medium, the slices absorbed it from the medium so that the amount found in the incubated slices was greatly elevated. If the weight of slices was sufficiently high relative to the volume of the medium this absorption of GABA resulted in a marked lowering of the concentration in the medium. The total amount of GABA estimated as Factor I in the slices plus medium was, within the limits of accuracy of the assays, unchanged. Representative results illustrating these points are shown in Table 3.

TABLE 4.—DISTRIBUTION OF γ-AMINOBUTYRIC ACID BETWEEN PARTICLES OF DISINTEGRATED CEREBRAL CORTEX AND THE SUSPENDING MEDIUM

The tissue suspension contained 75 mg tissue/ml

GABA in supernatant (μmoles/ml)		GABA in residue (μmoles/ml)	
Initial	Incubated	Initial	Incubated
0·1	0·12	2·16	2·2
1·1	1·2		1·9
2·1	2·3		2·4

Rat cerebral cortex was homogenized in the glucose-containing bicarbonate-buffered saline medium. A 3 ml sample of the suspension containing 225 mg tissue was centrifuged at 12,000 rev/min in a tared tube. The tube was weighed, the supernatant fluid was separated and its volume and that of the residue were determined by re-weighing the tube. The supernatant fluid was prepared for assay as described in the text for medium from slices; the residue, 0·3 ml, was suspended in tris-crayfish saline and heated before being assayed. Other 3 ml samples, containing the same amount of tissue with and without GABA additions, were incubated in Warburg vessels in the presence of oxygen–carbon dioxide and then treated in the same way.

Table 3 also shows representative results obtained with slices of kidney cortex and liver and sectors of diaphragm. None of these tissues appeared to absorb GABA against a concentration gradient. The concentrations found in these tissues after incubation with GABA-containing medium could be accounted for by simple diffusion from the medium into the tissue fluids.

The absorption of GABA by brain tissue is dependent upon structural integrity. Cerebral cortex slices were homogenized in the incubation medium and then GABA was added and the suspension was incubated. After centrifuging at high speed, the Factor I contents of the residue and of the supernatant fluid were assayed. Table 4 gives the results of a representative experiment which shows that the residue contained no more activity than the amount found originally occluded in the tissue. The activity in the supernatant fluid corresponded to the amount of GABA added plus a slight amount released from the tissue. The full activity in the supernatant fluid was found without heating and was therefore not in the occult form.

The rate of uptake of GABA by brain slices in indicated in Fig. 1 which shows that the absorption was rapid at first and continued more slowly for a considerable time. The initial rate of absorption by the slice may be limited by the rate of diffusion of GABA from the medium into the inner layers of the slice. The potential rate of absorption by the tissue itself may thus be greater than can be observed with slices.

Figs. 2a and 2b show the effect of the concentration of GABA in the medium on

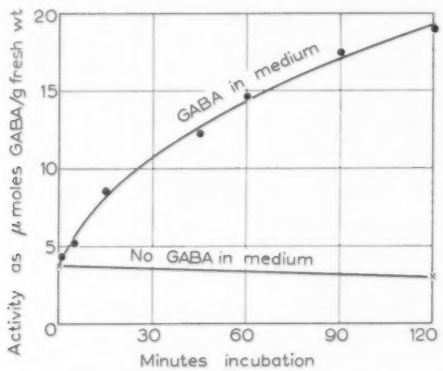

FIG. 1.—Time course of absorption of GABA from the medium by slices of cerebral cortex. The initial concentration of GABA in the medium was 2 μmoles/ml.

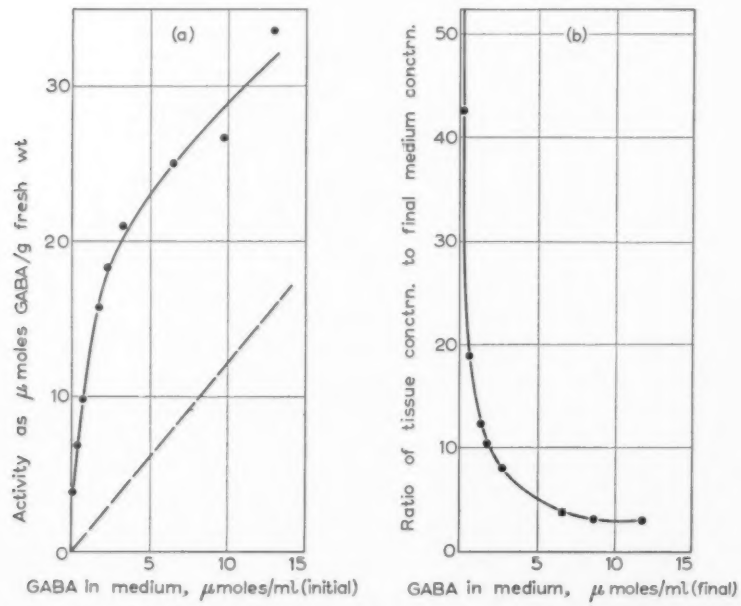

FIG. 2.—Effect of the concentration of GABA in the medium on the concentration of GABA in slices of cerebral cortex after 1 hr incubation. The broken line indicates the amount of GABA per gram fresh tissue which would be expected if the tissue fluids contained the same concentration of GABA as the suspending medium and no other uptake of GABA occurred. The incubated tissue is assumed to have swollen by 40 per cent and to contain 19 g solids and 121 g water/100 g of fresh tissue (PAPPIUS and ELLIOTT, 1956). (The volume of medium was 3 ml instead of the usual 2·5 ml.)

that in the tissue after incubation for 1 hr. The experiment illustrated is typical. Data obtained in numerous experiments fell on or near the curves shown. Fig. 2(a) shows that the amount of GABA absorbed per gram of slice increased with increasing concentration of GABA in the medium, especially with low concentrations in the medium. It is apparent from Fig. 2(b) that, when the medium contains little GABA, the ratio of the concentration in the tissue to that in the medium becomes extremely high.

The shape of the curve in Fig. 2(a) indicates that the GABA in the slice is in two fractions, a bound or occluded fraction, which reaches a maximum in the presence of about 5 mM in the medium, and a fraction which is in free solution in the water of the slice at the same concentration as in the medium. Back extrapolation indicates that the maximum concentration of GABA which can be occluded during 1 hr incubation is about 17 μmoles/g fresh weight.

It seems probable that the fraction of the GABA which becomes bound by the slices is in a similar condition to the GABA which is found in fresh unincubated tissue. Slices were homogenized as usual for assay and one part of the resulting suspension was assayed after the usual heating to give the total GABA and another part was assayed without heating to give an estimate of 'free' GABA; the difference was considered to be bound. Unincubated slices contained a total of 3·4 μmoles GABA/g of which 66 per cent was bound. Slices, from the same animal, after incubation for 1 hr in medium containing GABA (2 mM initial, 0·65 mM final) contained 14·7 μmoles/g of which 58 per cent was bound. It is likely that considerably more of the total activity in both unincubated and incubated slices was originally bound, but was liberated by the mechanical disruption of the slices.

Factors affecting the retention and uptake of GABA. When brain slices were incubated aerobically without glucose in the medium, their content of GABA decreased. The uptake of GABA by slices from GABA-containing medium was much less marked in the absence than in the presence of glucose. When slices were incubated anaerobically, with or without glucose present, they lost still more of their occluded GABA and measurable amounts of GABA could be found free in the medium. When GABA was added to the medium under anaerobic conditions apparently none was taken up into the occult form in the slice; about half of the GABA found in the slice could be accounted for as free GABA present in the fluids of the swollen slice in the same concentration as in the medium. Representative results showing these effects are given in Table 5.

The amount of GABA found in the slices incubated, with or without GABA added to the medium, was not obviously affected by increasing the potassium concentration of the medium from the usual 3·4 mM to 15 or to 40 mM. (The sodium concentration was reduced appropriately to maintain isotonicity.) The effects of variations in other cations have not been tested.

Production of GABA by slices. The decarboxylation of glutamate with the production of GABA has been shown to occur in brain tissue preparations (Awapara *et al.*, 1950, Roberts and Frankel, 1950, Wingo and Awapara, 1950) and to be dependent upon pyridoxal phosphate as coenzyme (Roberts and Frankel, 1950, 1951).

Figure 3 shows that, when cerebral cortex slices were incubated in medium containing added glutamate, the Factor I content of the slices increased up to a

TABLE 5.—EFFECT OF METABOLIC CONDITIONS ON OCCLUSION OF γ-AMINOBUTYRIC ACID BY CEREBRAL CORTEX SLICES

GABA added to medium (μmoles/ml)	Aerobic				Anaerobic			
	Glucose		No glucose		Glucose		No glucose	
	Tissue	Medium	Tissue	Medium	Tissue	Medium	Tissue	Medium
	5 min incubation							
0	3·0		2·7		2·7		2·7	
	10 min incubation							
0	3·4		3·6		2·7		2·7	
	60 min incubation							
0	3·6	<0·05	2·6	0·05	1·2		0·5	
2	13·6				3·8		4·0	
0	3·3	<0·03	1·6	0·03	1·2	0·15	1·2	0·22
2	14·3		8·0		4·1		3·8	

Figures show Factor I activity as μmoles GABA/g of fresh tissue or per ml of medium.

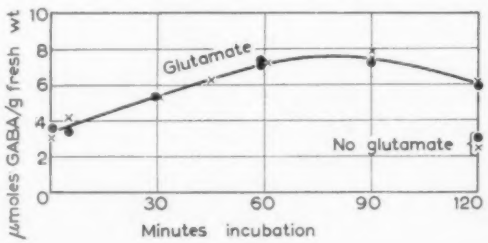

FIG. 3.—Time course of the effect of glutamate in the medium on the amount of Factor I in incubated slices of cerebral cortex. Initial glutamate concentration, 25 mM. The points marked ● and × were obtained in separate experiments.

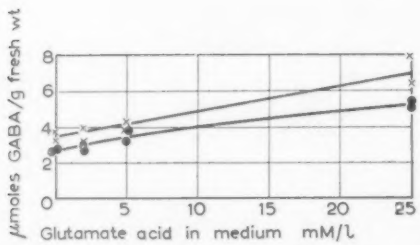

FIG. 4.—Effect of the concentration of glutamate in the medium on the Factor I content of incubated slices. Incubation time, 1 hr. The points marked ● and × were obtained in separate experiments.

maximum, which was reached after about 1 hr, and thereafter declined slowly. Figure 4 shows that the concentration of Factor I which is reached in the tissue depends upon the concentration of glutamate in the medium.

Representative results given in Table 6 show that the high concentration of Factor I which is found in slices after incubation in GABA-containing medium is not further increased by adding glutamate, as well as GABA, to the medium. This failure of glutamate to cause further increase in the GABA content occurred even when the amount of GABA in the slice was well below the maximum amount which can be occluded. There was no sign that extra GABA was being produced from glutamate and extruded into the medium.

DISCUSSION

BESSMAN, ROSSEN and LAYNE (1953) and ROBERTS and BREGOFF (1953) have shown that preparations from brain can catalyse a reversible transamination reaction between GABA and α-ketoglutarate which yields succinic semialdehyde and glutamate. Recently TSUKADA, NAGATA and TAKAGAKI (1957) have shown that guinea pig brain

TABLE 6.—EFFECT OF GLUTAMATE WITH AND WITHOUT γ-AMINOBUTYRIC ACID IN THE MEDIUM ON THE FACTOR I CONTENT OF INCUBATED SLICES OF CEREBRAL CORTEX

GABA added to medium (μmoles/ml)	No glutamate		Glutamate (25 μmoles/ml)	
	Tissue	Medium	Tissue	Medium
0	3·3	<0·02	8·1	
5	22·2	4·0	20·8	4·0
0	3·4		7·6	
2	12·4		13·0	
0	3·0	<0·05	6·2	<0·05
1	13·2	0·64	11·9	0·72
0	3·2	<0·03	6·5	<0·03
0·5	9·0	0·14	9·6	0·24

Figures show Factor 1 activity, as μmoles GABA/g of fresh tissue or per ml of medium, after incubation for 1 hr in the presence of oxygen and glucose. Each figure is the mean of results with two sets of slices.

slices cause a rapid evolution of $^{14}CO_2$ from GABA labelled with ^{14}C in the carboxyl group and they produced evidence that GABA undergoes transamination in slices. They also reported increased oxygen uptake when 10 mM-GABA was present in the suspending medium, particularly if glucose was also present though there was no increase in glucose utilization.* We were therefore surprised to find no evidence of rapid destruction of Factor I activity in suspensions or slices of brain. It is possible that though there is little net change in the amounts of GABA present in the system this may be due to a balance between the rates of formation and destruction of GABA. We obtained full recovery even when GABA was added to the medium; this would indicate that such a balance is not appreciably affected by variations in the concentration of GABA in the medium or absorbed in the tissue.

There appears to be a relationship between the concentrations of glutamic acid and GABA in brain tissue. STERN, EGGLESTON, HEMS and KREBS (1949) showed that brain slices accumulate glutamate actively. The tables of STERN *et al.* indicate that guinea pig cortex slices contained initially about 18 μmoles of glutamate/g and somewhat less after incubation without added glutamate. When increasing amounts of glutamate were added to the medium in which the slices were incubated, the concentration in the slices increased. When the medium contained 20 mM-glutamate

* With rat cerebral cortex slices under similar conditions we have not found any marked effect of GABA on oxygen uptake.

the concentration in the slices reached 32 μmoles/g, that is about twice the concentration found without glutamate added to the medium. In Fig. 4 it is seen that with rat cortex slices the concentration of GABA in the tissue would be approximately doubled when 20 mM-glutamate is present in the medium. STERN *et al.* also found that the rise in glutamate concentration in the slices reached a maximum after about 40 min incubation in glutamate-containing medium and then fell. In Fig. 3 it is seen that the GABA content of the slices also reached a maximum soon after this time and then fell. The postulated balance between the rates of formation and destruction of GABA could be due to a balance between the rate of decarboxylation of glutamate which yields GABA (ROBERTS and FRANKEL, 1950, 1951) and the rate of transamination of GABA with α-ketoglutarate. The α-ketoglutarate could be provided from carbohydrate by the Krebs cycle. There is no evidence as yet to explain the finding that, when GABA and glutamate were both added to the medium, no more GABA was found in the tissue than with GABA alone. It seems possible that the presence of excess GABA may prevent the uptake of glutamate by the tissue and so prevent the effect of the added glutamate on the production of GABA.

Our failure to find evidence for any rapid inactivation of Factor I activity by brain tissue seems to indicate that GABA can scarcely act as a transmitter substance for inhibitory impulses in a manner precisely similar to that of an excitatory transmitter like acetylcholine. Nevertheless there seems to be sufficient evidence that GABA, which is present in brain and can produce profound neurophysiological effects, is likely to be an 'action substance' of significance. The present study shows that, although there is apparently no mechanism which rapidly lowers the concentration of GABA (estimated as Factor I), there is a mechanism whereby GABA can be withdrawn from free solution into a condition in which it is probably inactive. It seems to us that the transamination reaction discovered by BESSMAN *et al.* (1953) and ROBERTS and BREGOFF (1953) is probably involved in the utilization of GABA in energy-yielding metabolism and perhaps in causing relatively slow variations in the concentration of GABA, but it appears unlikely that this reaction is concerned in any very rapid changes in GABA concentration which might affect neuronal activity.

It seems possible that both glutamate and GABA may be 'action substances' of neurophysiological importance and that a balance between these substances in the free or occluded forms may to some degree determine neuronal activity in the brain. Factors which affect the absorption (occlusion) of the two substances might determine the actual balance of their activities. Glutamate, administered *via* the carotid artery or intra-cisternally, has been shown by HAYASHI (1954) to produce convulsions in dogs. As mentioned in the Introduction there is ample evidence that GABA can exert marked inhibitory actions in the central nervous system. Though the amounts of glutamate injected by HAYASHI, and the concentrations of GABA which have been applied topically to the brain by PURPURA, GIRADO and GRUNDFEST (1957 *a*,*b*) and IWAMA and JASPER (1957) are high, the actual concentrations of these substances which reached the sensitive sites in the brain are unknown and may have been reasonably low. Further, it does not seem necessary to consider that actions which can be exerted by a substance can be of physiological significance only if these actions are exerted at very low concentrations. Both glutamate (see, e.g. STERN *et al.*, 1949) and GABA are present in brain in quite large amounts and considerable changes in the concentrations of their free and occluded forms are readily conceivable.

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The possibility of a balance of effects of glutamate and GABA is suggested also by observations on the live crayfish. BAZEMORE, ELLIOTT and FLOREY (1956) reported that injections of 2 μmoles of GABA into the crayfish haemocoele produced a flaccid condition. We have found that injection of 5 μmoles of glutamate causes the animal to go into a rigid tonic condition. FLOREY (1956) and MCLENNAN (1957) have shown that GABA causes inhibition of neuromuscular transmission in the crayfish and, as mentioned earlier, we have noted that local application of glutamate can cause contractions of crayfish muscle. The effects of injected GABA and glutamate in the crayfish are likely therefore to be the results of peripheral effects on the neuromuscular apparatus though the central nervous system may also be affected.

SUMMARY

Brief applications of glutamate can cause contraction of crayfish muscle and affect the activity of the stretch-receptor neuron. Factor I activity can be assayed in extracts containing glutamate by adding excess glutamate, 5 mM, to the wash fluid and dilution medium.

γ-Aminobutyric acid (GABA), determined by its Factor I activity, is not inactivated at an appreciable rate by brain suspensions nor by slices of brain or other tissues.

Slices of cerebral cortex absorb GABA from the suspending medium. When the concentration of GABA in the medium is low, the concentration in the slices can become more than forty times as high as in the medium. The absorbed GABA is mainly in the occult form which does not show Factor I activity until it is released. The absorption or retention of GABA by slices is decreased in the absence of glucose and nearly completely abolished in the absence of oxygen.

GABA is not absorbed from the suspending medium by diaphragm sectors or by slices of liver or kidney nor by disintegrated brain tissue.

The Factor I (presumably GABA) content of brain slices is about doubled by incubation in the presence of 25 mM glutamate. When glutamate and GABA (0·05 mM or more) are both present in the medium, no more Factor I is found in the slice than with GABA alone.

The possibility that a balance between free glutamate and GABA may influence neuronal activity in the central nervous system is discussed.

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THE EFFECT OF AGE ON THE NON-HAEMIN IRON IN THE HUMAN BRAIN

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IRON is found in the normal brain as haemin iron in haemoglobin and in iron-containing enzymes and as non-haemin iron. At least part of the non-haemin iron can be shown by histochemical methods, the prussian blue and Turnbull blue reactions being most commonly used to demonstrate it. GUIZZETTI (1915) examined the iron reactions seen when thick, macroscopic brain sections were treated with potassium ferrocyanide or ammonium sulphide. He found that certain parts of the brain gave a stronger staining reaction than others: the globus pallidus, substantia nigra, red nucleus and dentate nucleus were most intensely stained. The most extensive and systematic investigations of histologically demonstrable iron in the brain were conducted by SPATZ (1922). Using thick, macroscopic sections SPATZ was able to divide the centres of the central nervous system into four groups according to their iron content.

The first group comprised the globus pallidus and substantia nigra, which gave the most intense and invariable iron reaction. The red nucleus, putamen, caudate nucleus, dentate nucleus and the subthalamic body, with constant but somewhat weaker staining properties, formed the second group. The structures included in the first and second groups belong to the so-called extrapyramidal system. The third group, which included the cerebral and cerebellar cortex, the anterior nucleus of the thalamus, the mamillary body, the tectum of the midbrain and the central grey matter of the third ventricle, gave a variable and considerably weaker reaction for iron. The centres of the fourth group showed no histochemical iron staining; it comprised the medulla oblongata, the grey matter of the spinal cord, the spinal and sympathetic ganglia and the white matter of the brain and the spinal cord. SPATZ also observed that the foetal brain never showed a positive iron reaction. In the first years of life iron could be demonstrated in the extra-pyramidal system and the amount gradually increased until puberty.

In microscopical sections from the centres of the two first groups SPATZ observed a diffuse iron reaction visible to the naked eye. He found a fine granular deposit of iron in individual oligodendroglia cells and nerve cells, mainly in the globus pallidus and in the red zone of substantia nigra. These granules could be observed only by using high magnification. HADFIELD (1929) stated that the walls of the blood vessels of the healthy globus pallidus from the age of 30 onward are often infiltrated with iron salts.

GELLERSTEDT (1933), when studying the alterations of the brain during normal involution, found granular iron deposits in the motor- and occipital cortex of individuals over 65 years of age without nervous or mental disease. These iron deposits were observed only in extremely small amounts in individual oligodendroglia cells,

endothelial cells of capillaries and giant pyramidal cells of the motor cortex. He also observed a positive iron reaction in the subcortical region of the white matter.

CUMINGS (1948) determined quantitatively the total iron, both haemin and non-haemin, in ashed samples from different parts of six normal brains. The highest values for total iron were found in the globus pallidus; then followed the putamen, caudate nucleus, thalamus, cortical grey and cortical white matter. STEIN (1923) tried to estimate the non-haemin iron by determining the total iron after exsanguination of the brain. In the single case examined, the extrapyramidal system was found to contain twice as much iron as the frontal cortex. TINGEY (1937, 1938) extracted the non-haemin iron with sodium pyrophosphate. The extraction with pyrophosphate may be incomplete, but TINGEY used a long extraction time and this seems to give more reliable results. A few cases of brains from adults and children were examined. Less iron was found in the brains of children than in those of adults; normal figures were reached at eleven years.

The effect of age on the contents of non-haemin iron in different parts of the brain has so far not been systematically studied. In the present paper diagrams demonstrating the relationship between age and the quantities of non-haemin iron in the different regions of the brain are presented. This knowledge is essential when investigating the non-haemin iron in pathological conditions of the brain. The amounts of iron demonstrable by histochemical staining methods are compared with the figures obtained from quantitative determinations. There is no conclusive evidence about the forms in which the non-haemin iron in the brain exists. DIEZEL (1955) maintains that at least a part of it is present as ferritin.

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MATERIAL AND METHODS

In this study, unfixed material was used from brains of eighty-one patients who died at the Royal Academic Hospital, Uppsala. The brains of patients with cerebrovascular and neuropsychiatric disorders were carefully excluded. In addition seventeen brains of young persons dying from accidents were also examined.

Chemical determination of non-haemin iron. The non-haemin iron was determined by the method developed by HALLGREN (1953). The non-haemin iron is extracted from homogenized tissue samples with 5 N-hydrochloric acid at 80–90°C for 1 hr. This extraction method fulfils the condition of not liberating more than insignificant quantities of haemin iron. The iron is then determined colorimetrically by the *ortho*phenanthroline method. The extinctions are estimated by means of a Beckman B spectrophotometer at 500 mμ. The iron content is calculated on a fresh weight basis. The dry weight of the samples was also determined in order to exclude cases of excessive oedema of the brain.

Histopathological methods. SPATZ (1922) found the Turnbull blue method to be more sensitive and reliable than the prussian blue method for demonstrating iron in nervous tissue. We have confirmed this. The specimens were fixed in 96% ethanol, embedded in paraffin and cut at 20 μ. For diagnostic purposes the toluidine blue staining method for nerve cells and Palmgren's silver method for nerve fibres were used.

RESULTS AND COMMENTS

Distribution of Non-haemin Iron in the Brain

The distribution of non-haemin iron in different parts of the adult brain is shown

TABLE 1(a).—THE DISTRIBUTION OF NON-HAEMIN IRON IN DIFFERENT PARTS OF THE HUMAN BRAIN AUTOPSY CASES, 30–100 YEARS OF AGE

	No.	mg iron/100 g fresh weight	$\pm\sigma$
Globus pallidus	55	21·30	3·49
Red nucleus	44	19·48	6·86
Substantia nigra	52	18·46	6·52
Putamen	56	13·32	3·43
Dentate nucleus	45	10·35	4·86
Caudate nucleus	58	9·28	2·14
Thalamus	52	4·76	1·16
Cerebellar cortex	53	3·35	0·87
Motor cortex*	46	5·03	0·88
Occipital cortex	38	4·55	0·67
Sensory cortex	45	4·32	0·58
Parietal cortex	37	3·81	0·67
Temporal cortex	47	3·13	0·57
Prefrontal cortex	58	2·92	0·41
Frontal white matter	59	4·24	0·88
Medulla oblongata	41	1·40	1·16
Meninges	10	1·02	0·29
Liver	37	13·44	9·36

* 40–100 years of age.

TABLE 1(b).—COMPARISON BETWEEN DIFFERENT PARTS OF THE HUMAN BRAIN

	Difference	t	P
Globus pallidus—substantia nigra	2·84	2·79	<0·01
Substantia nigra—putamen	5·14	5·07	<0·001
Putamen—caudate nucleus	4·04	4·03	<0·001
Putamen—dentate nucleus	2·97	3·47	<0·001
Dentate nucleus—caudate nucleus	1·07	0·93	<0·4
Motor cortex—occipital cortex	0·48	2·82	<0·01
Motor cortex—sensory cortex	0·71	4·55	<0·001
Occipital cortex—parietal cortex	0·74	4·77	<0·001
Parietal cortex—temporal cortex	0·68	4·92	<0·001
Frontal white matter—prefrontal cortex	1·32	10·49	<0·001

in Table 1 (a). The average content of non-haemin iron in different regions of the brain has been calculated in persons between 30–100 years of age. The highest iron content is found in the centres of the extrapyramidal system. The maximum amount occurs in the globus pallidus with an average of 21·30 mg non-haemin iron per 100 g fresh weight. Somewhat lower figures are obtained for the red nucleus and substantia nigra. The difference between globus pallidus and substantia nigra is significant ($P <$ 0·01). Fourth in order is the putamen with an average of 13·32 mg of non-haemin iron per 100 g fresh weight. Next in order follow the dentate nucleus and caudate nucleus.

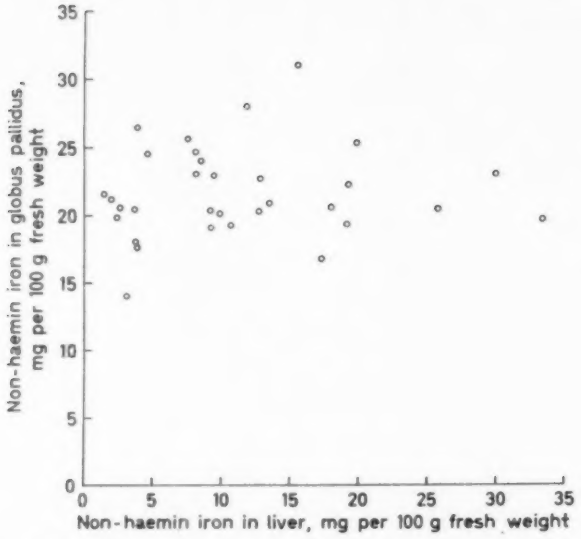

FIG. 1. The relationship between non-haemin iron in the globus pallidus and liver.

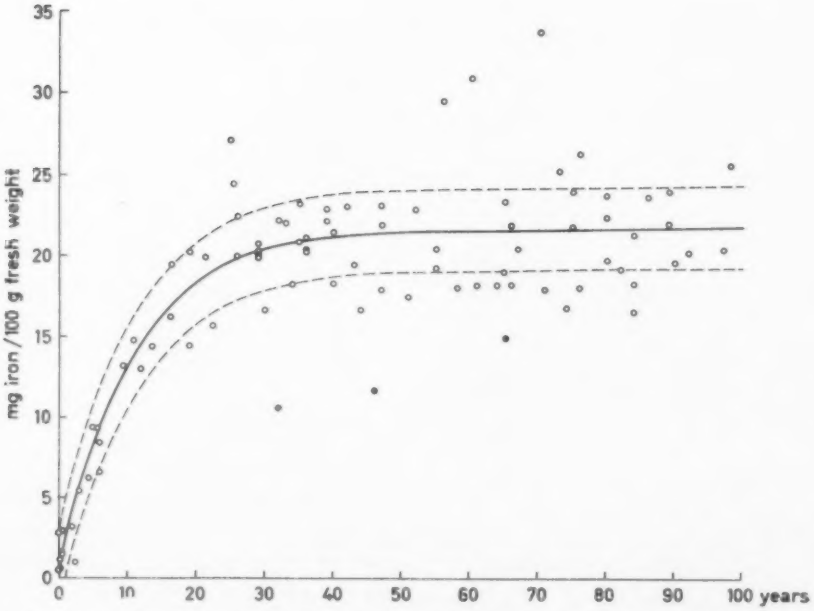

FIG. 2. Non-haemin iron in the globus pallidus at different ages. The filled circles represent cases with large intestinal haemorrhages.
The calculated regression lines have been drawn in Figs. 2–6. The dotted lines denote the the S.E. of estimate; for globus pallidus S.E. = ±3·07.

The distribution of non-haemin iron in different cortical fields is as follows (see Table 1(a)). The motor cortex (cortex of the precentral gyrus) has the highest content with a mean content of 5·03 mg iron per 100 g fresh weight. This is closely followed by the occipital cortex, the sensory cortex (postcentral gyrus) and then the rest of the parietal cortex. The difference between, for example, motor cortex and sensory cortex is highly significant (see Table 1(b)). The phylogenetically young cortical fields, namely the prefrontal cortex and the neopallial part of the temporal cortex, show the lowest iron values.

In the corpus striatum less iron is found in the phylogenetically younger centres (putamen + caudate nucleus) than in the phylogenetically older globus pallidus. This relationship between phylogenetic age and the content of non-haemin iron is also valid on comparing the extrapyramidal system and the cerebral cortex at large. In the white matter both microscopic and macroscopic iron reactions are negative. In spite of this, the frontal white matter contains more non-haemin iron than the corresponding cortex, 4·24 and 2·92 mg iron per 100 g fresh weight respectively ($P < 0{\cdot}001$). Only small quantities of non-haemin iron are found in medulla oblongata and the leptomeninges, 1·40 and 1·02 mg per 100 g fresh weight respectively. For the sake of comparison the iron content of the liver is included in Table 1(a). The mean value for storage iron in the liver of thirty-seven patients examined amounts to 13·44 mg per 100 g fresh weight. A liver well charged with iron contains about 15–20 mg non-haemin iron per 100 g fresh weight, the total amount being 250–300 mg. The whole brain has been estimated to contain about 60 mg non-haemin iron, i.e. approximately one-fifth of the content of a well charged liver. On comparison it is evident that the iron content of the extrapyramidal centres having the highest iron concentration is of the same magnitude as that of a liver well charged with storage iron.

The quantities of non-haemin iron in the different parts of the brain seems to be largely independent of the amounts of storage iron in the iron depots. This is demonstrated in the case of the globus pallidus in Fig. 1, where the non-haemin iron values are plotted against those of the liver. The same is true for the other structures examined. In cases with a scanty supply of storage iron the brain may contain more non-haemin iron than the liver.

The Non-haemin Iron at Different Ages

Extrapyramidal system and thalamus. Figure 2 demonstrates the relationship between age and the non-haemin iron content of the globus pallidus. At birth very low iron values are found; then follows a rapid increase during the first two decades. No further increase seems to occur after 30 years of age. In the putamen (Fig. 3) and caudate nucleus the iron content increases somewhat more slowly, the maximum values not being reached before the age of 50–60 years. The iron values of red nucleus, substantia nigra and dentate nucleus show a considerable scattering; this is at least partly due to the difficulties in delimiting these structures at dissection. A rapid increase in non-haemin iron content during the two first decades is, however, clearly demonstrated also for these structures.

Cerebral and cerebellar cortex. In Fig. 4 the age correlation of non-haemin iron in motor cortex is given. As seen from the diagram, the iron content increases with age up to about 50 years. In prefrontal cortex (see Fig. 5) the increase in iron value proceeds very slowly or perhaps not at all after 30 years of age. This seems also to be true for

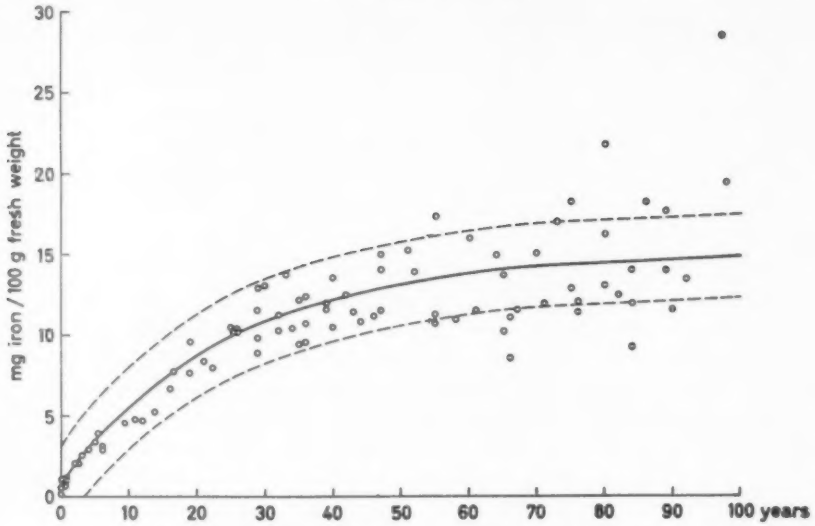

FIG. 3. Non-haemin iron in the putamen. S.E. of estimate = ±2·60.

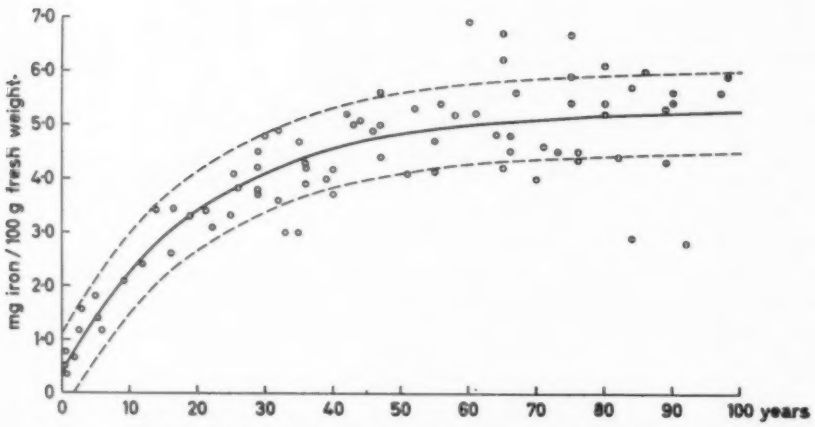

FIG. 4. Non-haemin iron in the motor cortex. S.E. of estimate = ±0·74.

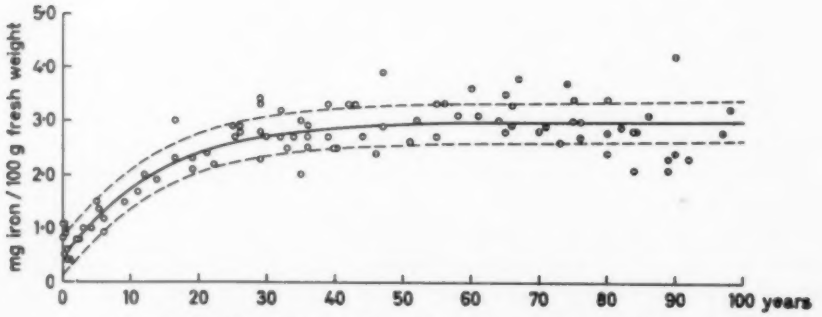

FIG. 5. Non-haemin iron in the prefrontal cortex. S.E. of estimate = ±0·37.

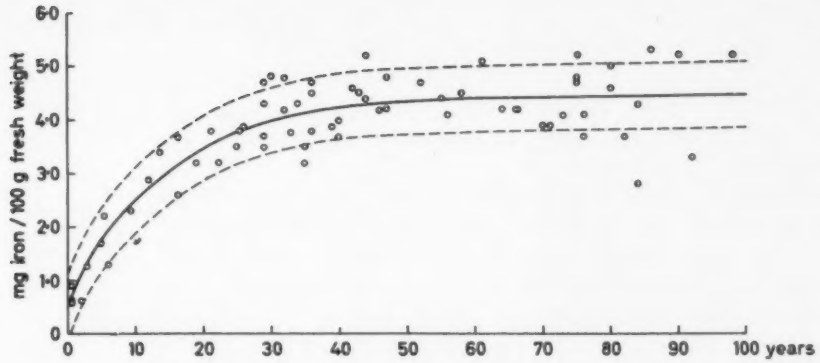

FIG. 6. Non-haemin iron in the sensory cortex. S.E. of estimate = ±0·60.

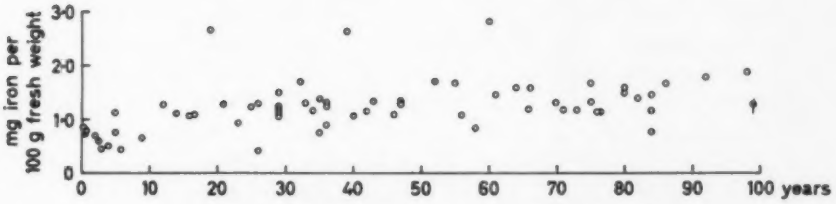

FIG. 7. Non-haemin iron in the medulla oblongata.

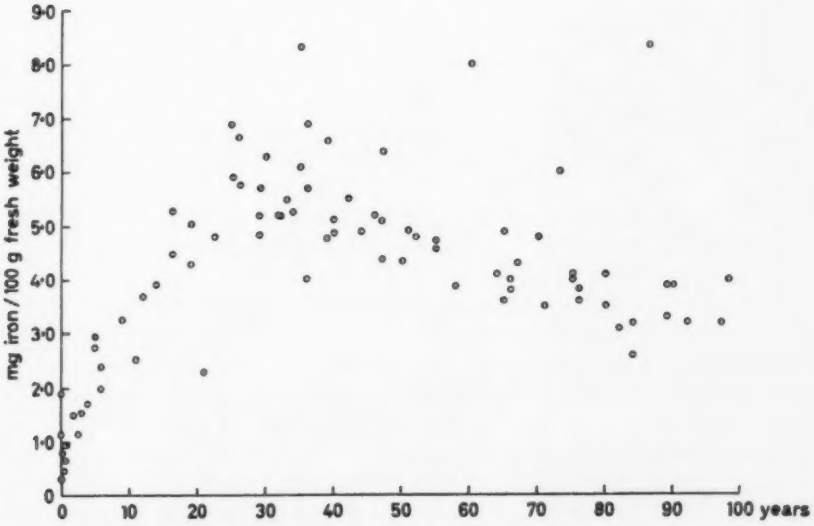

FIG. 8. Non-haemin iron in the thalamus.

the sensory (Fig. 6) and the cerebellar cortex. In the case of cerebellum the specimens were taken from the neocerebellar part of the hemispheres.

White matter. White matter was taken from the frontal part of centrum semiovale. In adults the concentration of non-haemin iron was higher in white than in grey frontal matter. The rise in content during childhood and adolescence is also more rapid in the white matter.

Medulla oblongata. Sections 1 cm thick were taken for analysis from the caudal end of the medulla oblongata including the inferior olives and the area postrema. No increase in iron values with age is found (see Fig. 7). Thus the medulla oblongata is an exception to the rule that the iron content increases with age.

Thalamus. The analyses performed on thalamus (Fig. 8) revealed a rise in non-haemin iron content up to about 35 years. Then there seems to be a decline in iron values.

If the regression lines are calculated according to the method of least squares we obtain the following equations:

Frontal white matter	$y = 3{\cdot}95\,\{1 - \exp(-0{\cdot}10x)\} + 0{\cdot}31$
Globus pallidus	$y = 21{\cdot}41\,\{1 - \exp(-0{\cdot}09x)\} + 0{\cdot}37$
Cerebellar cortex	$y = 2{\cdot}70\,\{1 - \exp(-0{\cdot}085x)\} + 0{\cdot}68$
Prefrontal cortex	$y = 2{\cdot}43\,\{1 - \exp(-0{\cdot}07x)\} + 0{\cdot}58$
Temporal cortex	$y = 2{\cdot}70\,\{1 - \exp(-0{\cdot}07x)\} + 0{\cdot}55$
Sensory cortex	$y = 3{\cdot}97\,\{1 - \exp(-0{\cdot}07x)\} + 0{\cdot}49$
Parietal cortex	$y = 3{\cdot}31\,\{1 - \exp(-0{\cdot}06x)\} + 0{\cdot}60$
Occipital cortex	$y = 4{\cdot}03\,\{1 - \exp(-0{\cdot}06x)\} + 0{\cdot}72$
Motor cortex	$y = 4{\cdot}79\,\{1 - \exp(-0{\cdot}05x)\} + 0{\cdot}40$
Caudate nucleus	$y = 9{\cdot}66\,\{1 - \exp(-0{\cdot}05x)\} + 0{\cdot}33$
Putamen	$y = 14{\cdot}62\,\{1 - \exp(-0{\cdot}04x)\} + 0{\cdot}46$

y = mg non-haemin iron per 100 g fresh weight
x = age in years

The coefficients of x, i.e. −0·10, −0·09, −0·07, etc., constitute a measure of the slope of the regression lines. For large negative coefficients, the slopes of the regression lines are more steep and their approach to a limiting value more rapid. For comparison the regression lines of the different cortical fields have been compiled in Fig. 9.

Histochemical estimations of non-haemin iron. Since non-haemin iron may originate from vascular lesions, it is essential to make a thorough search for haemorrhages. Before accepting the brain as normal, macroscopic and microscopic haemorrhagic lesions must be absent.

The quantitative distribution of non-haemin iron in the extrapyramidal system conforms fairly well with the histological estimations. Minor differences in iron content between the globus pallidus and substantia nigra are, however, impossible to detect by histological methods. The substantia nigra is found to contain more histologically demonstrable iron than the red nucleus. As can be seen from Table 1(a), the two structures contain about the same amount of non-haemin iron. The granular deposit in nerve cells and glia cells of the substantia nigra may be the cause of the strong iron reaction. The red nucleus gives a diffuse iron staining, which is less striking.

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The cortex of normal brains from individuals less than 65 does not show histologically detectable iron. In old people minimal amounts of such iron appear in the occipital and motor cortex. Up to the present we have not been able to find iron in histological sections from the frontal, temporal and cerebellar cortex of normal brains. (The phylogenetically old parts of the frontal and temporal lobes have not so far been investigated). The amounts of iron seen in the occipital and motor cortex of old

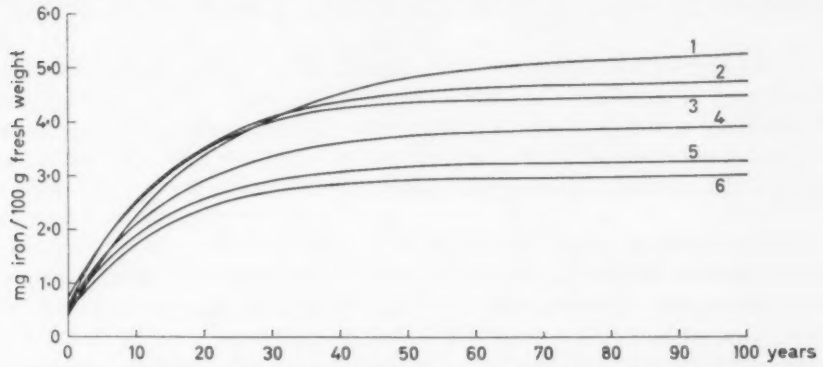

FIG. 9. Relation between the non-haemin iron and the age in different cortical fields. (1) motor cortex; (2) occipital cortex; (3) sensory cortex; (4) parietal cortex; (5) temporal cortex; (6) prefrontal cortex.

brains roughly parallel the occurrence of yellow pigment (lipofuscin) in nerve and glia cells. In the white matter and medulla oblongata no iron deposits are found.

The binding of the non-haemin iron

The brains from three dogs (2, 5 and 7 years), were homogenized in 0·25 M-sucrose. Fractionation according to the method of HOGEBOOM and SCHNEIDER (1948) yielded a nuclear fraction, mitochondrial fraction, microsomal fraction and a soluble fraction. Non-haemin iron was found in all cell fractions. The mitochondria and microsomes together contain almost half of the total non-haemin iron (46 per cent on the average). About 14 per cent is found in the nuclei and the remaining 40 per cent in the soluble fraction. The microsomes had the highest iron content calculated on a dry weight basis, about 4 mg per 100 g. The mitochondria contained about 2 mg and the nuclei 0·8 mg per 100 g dry weight. For the sake of comparison, we have noted that the liver mitochondria of the rat contain about 13 mg non-haemin iron per 100 g dry weight.

About one-third of the non-haemin iron of the human brain could be extracted from homogenized tissue with pH 4·5 sodium acetate buffer. The extraction was continued for 12 hr, using 2 ml acetate buffer solution per 1 gram fresh tissue. After centrifugation a clear brownish-red supernatant could be decanted. To this extract was added acetone, precooled to −20°, to a proportion of 45 per cent v/v and the mixture let stand for 12 hr. The precipitate was collected by centrifugation and was found to contain the non-haemin iron. The precipitate was lyophilized, then dissolved in a few millilitres of acetate buffer and dialysed against distilled water. After lyophilization of the solution a dry powder with a non-haemin iron content of 10–12 mg iron per gram was obtained. This powder consisted mainly of protein which was soluble in distilled water. A concentrated solution had a strong brown colour.

In paper electrophoresis the iron-containing protein migrated as a β-globulin. The iron was detected by the prussian blue method. In zone electrophoresis the movement of the brown protein could be followed. We used a barbiturate buffer, pH 8·6, ionic strength 0·1 N and a potato starch block as supporting medium. The spreading of the zone was, however, considerable and no further purification was achieved. It was observed that the brown colour and the iron content of the fractions paralleled each other closely. A further enrichment of the iron could be achieved by fractionation with ammonium sulphate. The protein precipitating between 40 and 50 per cent saturation at pH 6 had non-haemin iron content as high as 3 per cent.

The high iron content, the solubility, the electrophoretic mobility and the degree of stability towards heat (80°) make it probable that the soluble iron-containing protein in the brain is similar to if not identical with ferritin. We have not been able to crystallize ferritin from human, horse or cow brain with cadmium sulphate.

DISCUSSION

Part of the non-haemin iron in the brain is present as an iron-rich protein which seems to be similar to ferritin. This iron can probably be regarded as storage iron reserved for the iron requirements in the brain.

It has been stated in this paper that the quantity of non-haemin iron in the brain is to a high degree independent of the iron stores of the body. However in three cases of ulcerating intestinal carcinomas associated with large haemorrhages somewhat lowered values for non-haemin iron in the extrapyramidal system have been observed. Further, in a case of severe anaemia lowered values were also found in the cortical areas. These observations suggest that in cases of depleted iron stores the non-haemin iron in the brain may be partly mobilized for metabolic needs outside the brain.

KETY (1955) has studied the oxygen consumption of the human brain at different ages. When comparing the age correlations for non-haemin iron and for oxygen consumption, it is evident that the rapid rise in non-haemin iron during the first decades corresponds to a rapid decline in oxygen utilization. In the following period the more gradual fall in oxygen consumption is paralleled by a slower increase in non-haemin iron. In fact the curves appear to be reciprocal. It has been suggested that non-haemin iron might participate in the electron transport system of the mitochondria (MAHLER, 1955). A high concentration of non-haemin iron in brain mitochondria (as well as in the microsomes) has been found in our study. The increase in non-haemin iron in the brain during maturation and ageing suggests that this may reflect an alteration in the enzyme systems of the cell.

SUMMARY

(1) The content of non-haemin iron in different parts of the human brain has been determined from birth to old age. The highest iron values were found in the structures of the extrapyramidal system.

(2) The distribution of the non-haemin iron in the cerebral cortex shows a characteristic pattern. The highest concentrations were found in the motor cortex, followed by occipital, sensory and parietal cortex. The lowest figures were found for the prefrontal and temporal cortex.

(3) An increase in non-haemin iron occurs with advancing age in all regions of the brain examined except in the medulla oblongata. The rise in iron values is rapid

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during the first two decades of life, then more gradual. There are some differences in the slopes of the curves for the structures investigated.

(4) The quantity of non-haemin iron in the brain is to a large extent independent of the iron stores in the body.

(5) A parallel is found between iron content and phylogenetic age of the structures investigated.

(6) The histological estimations of the amount of iron in the extrapyramidal centres agree fairly well with the chemical determinations. In the cerebral cortex histologically demonstrable iron could not be found in young and middle aged individuals. After the age of about 65 minimal iron deposits were detected in the motor and occipital cortex. No microscopically visible iron was found in the white matter.

(7) About one-third of the non-haemin iron of the brain can be extracted by buffer solutions. This iron is bound to a protein which seems to be similar to ferritin. It is assumed that this iron-rich protein is a store of iron reserved for the needs of the brain.

(8) A high concentration of non-haemin iron is found in microsomes and mitochondria of dog brain.

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CHEMICAL STUDIES OF PERIPHERAL NERVE DURING WALLERIAN DEGENERATION*—VII

LABELLING OF PHOSPHOLIPID AND CHOLESTEROL FROM [*CARBOXY*-^{14}C] ACETATE

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As the result of electron microscopic studies of developing peripheral nerve, GEREN (1954) has shown convincingly that the Schwann cell is responsible for the formation of the myelin sheath and that the myelin sheath quite properly may be regarded as an integral part of this cell. It was thus of interest to study the capability of a peripheral nerve to form some of the known components of the myelin sheath under conditions where there is an increase in the number of Schwann cells along the course of the nerve. The number of Schwann cells may be increased by sectioning the nerve, for it is known that a proliferation of Schwann cells forms part of the series of changes that occurs during classical Wallerian degeneration.

Since phospholipid and non-esterified cholesterol are included among the myelin lipids (JOHNSON, MCNABB and ROSSITER, 1950), the effect of either nerve section or nerve crush on the subsequent *in vitro* labelling of these lipids from [*carboxy*-^{14}C] acetate was studied. It was found that when the number of Schwann cells was increased as a result of either of these procedures, the labelling of both phospholipid and cholesterol was increased. Previously a similar increase was described for the labelling of phospholipid from inorganic ^{32}P (MAGEE and ROSSITER, 1954).

Preliminary accounts of some of these experiments have appeared (ROSSITER, 1957; ROSSITER, PRITCHARD and STRICKLAND, 1958).

METHODS

The right sciatic nerve of a number of cats was either sectioned or crushed at the level of the greater trochanter of the femur. Details of the operations have been described previously (JOHNSON, MCNABB and ROSSITER, 1949; BURT, MCNABB and ROSSITER, 1950). In the sectioned nerves adequate precautions were taken to ensure that no regeneration occurred. No attempt was made to control the age, sex or weight of the animals, since the sciatic nerve of the opposite side was left intact to serve as a control. After 8, 16, 32, 96, or 632 days the animals were killed and the segment of the nerve distal to the site of operation was removed, together with a similar length of intact left sciatic nerve. Each nerve was cleaned of adherent fatty and connective tissue; the epineurium was stripped off and the nerve was rapidly weighed. It was cut in small lengths (about 1 mm) and transferred to a Warburg flask containing Krebs–Ringer bicarbonate buffer to which had been added 3 μmoles [*carboxy*-^{14}C] acetate containing 1·3 μc radioactivity. The final volume was 5 ml. The flask was attached to its manometer and gassed with a 95% O_2–5% CO_2 gas mixture. The sample was then incubated for 4 hr at 37·5° with shaking to ensure adequate oxygenation.

After the incubation the nerve fragments were transferred to a mortar and the excess liquid was poured off. They were then frozen with liquid nitrogen and ground to a fine powder. The powder

* References to previous papers in the series may be found in MAGEE and ROSSITER (1954).

was extracted successively with three 5 ml portions of cold ethanol, four 5 ml portions of hot ethanol–ether 3 : 1 v/v and one 5 ml portion of ethyl ether. The lipid extracts were pooled and taken to dryness at 40–50° with reduced pressure under an atmosphere of nitrogen. The residue was dissolved in ethyl ether and radioactive water-soluble compounds were removed by washing with 5 ml portions of 0·05 M-sodium acetate carrier in 2 M-sodium chloride. Phospholipid was precipitated by the addition of acetone and non-esterified cholesterol was precipitated with digitonin as described by KLINE and DELUCA (1956).

Suitable portions of the phospholipid and cholesterol extracts were plated on weighed aluminium planchets and taken to dryness by rotation under an infra-red lamp. The radioactivity of the samples was then determined in a windowless gas-flow counter (Nuclear Instrument and Chemical Corporation, Chicago). The amount of phospholipid was determined gravimetrically and the amount of cholesterol was determined in another portion of the cholesterol extract by the method of SPERRY and WEBB (1950). Net counting rates were corrected for self-absorption and were adjusted to a standard amount of radioactivity added to the incubating medium. The standard error of counting was maintained below the 5 per cent level.

With [*carboxy*–^{14}C] acetate, and some other ^{14}C-labelled precursors, the specific radioactivity of the lipid precipitated by acetone was found to be lower if the tissue was treated with trichloroacetic acid before the lipid extraction. Experiments are now in progress to determine whether the treatment with trichloroacetic acid removed non-lipid contaminants, or whether it destroyed labile lipid components, or both. In any event, the observed increase in degenerating nerves was similar, whether the tissue was treated with trichloroacetic acid or not.

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RESULTS

Normal nerves. Table 1 shows the specific radioactivity of the phospholipid of normal sciatic nerve of the cat after incubation for 4 hr in the medium containing [*carboxy*–^{14}C] acetate. In each of the two experiments, the right and the left sciatic nerve were incubated separately. The table illustrates the degree of reproducibility to be expected in experiments of this type.

TABLE 1.—LABELLING OF THE PHOSPHOLIPID OF THE SCIATIC NERVE OF THE CAT

Nerve fragments incubated at 37·5° in Krebs–Ringer bicarbonate containing [*carboxy*–^{14}C] acetate. Incubation time, 4 hr.

Expt. No.	Specific radioactivity (counts/min per mg phospholipid)	
	Right nerve	Left nerve
1	1900	2310
2	860	720

Sectioned nerves. Table 2 shows that there was no consistent change in the specific radioactivity of the phospholipid in nerves that had been allowed to degenerate for 8 to 16 days and then incubated in the medium containing [*carboxy*–^{14}C] acetate. However, the specific radioactivity of the phospholipid in the nerves that were studied 32, 96, or 632 days after the operation was much greater than that of the intact control nerve of the opposite side.

It is well known that there is a progressive fall in the concentration of phospholipid in nerves degenerating after section. Using the data of JOHNSON *et al.* (1949),

TABLE 2.—EFFECT OF NERVE SECTION ON THE LABELLING OF THE PHOSPHOLIPID OF THE SCIATIC NERVE OF THE CAT

Nerve fragments incubated at 37·5° in Krebs–Ringer bicarbonate containing [*carboxy*-^{14}C] acetate. Incubation time, 4 hr.

Time after section (days)	Specific radioactivity (counts/min per mg phospholipid)		Radioactivity (counts/min per 100 mg wet tissue)	
	Intact control	Sectioned	Intact control	Sectioned
8	290	220	1750	1340
8	460	570	2780	3420
16	780	1360	4710	4060
16	770	790	4640	2360
32	430	2390	2590	3110
32	1350	3350	8150	4350
96	430	3640	2590	2190
96	210	14500	1270	8700
632	110	3200	680	1920

the number of counts per minute due to labelled phospholipid per 100 mg nerve was calculated. Because of the fall in the concentration of phospholipid in the degenerating nerves, the amount of radioactivity due to labelled phospholipid per 100 mg nerve was not consistently different from that in the intact control nerves at any of the time intervals studied.

The figures for non-esterified cholesterol are shown in Table 3. In the nerves studied 32 days after section the specific radioactivity of the cholesterol was of the same order as that of the control nerves from the opposite side, but by 96 days there was a great increase.

There is also a progressive fall in the concentration of non-esterified cholesterol as the degeneration proceeds. When this decrease was allowed for, using data of JOHNSON *et al.* (1949), the number of counts per minute due to labelled cholesterol

TABLE 3.—EFFECT OF NERVE SECTION ON THE LABELLING OF THE CHOLESTEROL OF THE SCIATIC NERVE OF THE CAT

Nerve fragments incubated at 37·5° in Krebs–Ringer bicarbonate containing [*carboxy*-^{14}C] acetate. Incubation time, 4 hr.

Time after section (days)	Specific radioactivity (counts/min per mg cholesterol)		Radioactivity (counts/min per 100 mg wet tissue)	
	Intact control	Sectioned	Intact control	Sectioned
32	130	100	420	115
32	120	170	380	195
96	150	4960	480	1440
96	145	6400	460	1860

per 100 mg nerve for the 32-day degenerating nerves was found to be less than the corresponding value for the intact control nerves. On the other hand, by 96 days the amount of radioactivity due to labelled cholesterol per 100 mg nerve was considerably greater.

Crushed nerves. Table 4 shows the results for four animals in which the nerve had been crushed rather than sectioned 96 days previously. In each instance the specific radioactivity of both the phospholipid and the non-esterified cholesterol in

TABLE 4—EFFECT OF NERVE CRUSH ON THE LABELLING OF THE PHOSPHOLIPID AND CHOLESTEROL OF THE SCIATIC NERVE OF THE CAT

Nerve fragments incubated at 37·5° in Krebs–Ringer bicarbonate containing [*carboxy*–^{14}C] acetate. Incubation time, 4 hr. Time after operation, 96 days.

Phospholipid				Cholesterol			
Specific radioactivity (counts/min per mg phospholipid)		Radioactivity (counts/min per 100 mg wet tissue)		Specific radioactivity (counts/min per mg cholesterol)		Radioactivity (counts/min per 100 mg wet tissue)	
Intact control	Crushed	Intact control	Crushed	Intact control	Crushed	Intact control	Crushed
380	2970	2290	6410	150	1800	480	1980
510	3150	3080	6800	150	1500	480	1650
800	8250	4820	17,800	120	3980	380	4380
500	16,700	3020	36,100	95	5130	300	5640

the regenerating nerve was considerably greater than the corresponding figure for the intact nerve of the opposite side. The increases in specific radioactivity were of the same order as those reported for sectioned nerves (Table 2 for phospholipid; Table 3 for cholesterol). However, in nerves regenerating after crush the concentration of both phospholipid and non-esterified cholesterol was greater, because of the considerable degree of remyelination that had taken place by this time (BURT *et al.*, 1950). As a result, the radioactivity due both to labelled phospholipid and to labelled non-esterified cholesterol per 100 mg nerve for the regenerating nerves was considerably greater than that for the intact control nerves, or than that for the nerves degenerating after section.

DISCUSSION

Phospholipid. The experiments show that even for the intact control nerves there was a definite *in vitro* labelling of phospholipid when fragments of the nerve were incubated in a suitable medium containing [*carboxy*–^{14}C] acetate. The phospholipid of peripheral nerve is also labelled from inorganic ^{32}P (FRIES, SCHACHNER and CHAIKOFF, 1942; MAGEE and ROSSITER, 1954) and from other ^{14}C-containing precursors such as glycerol, choline, ethanolamine and serine (ROSSITER, 1957; ROSSITER *et al.*, 1958).

It is well known that the phospholipid of brain and spinal cord is poorly labelled *in vivo* from a wide range of radioactive precursors, including [*carboxy*–^{14}C] acetate (see ROSSITER, 1957). However, under *in vitro* conditions, the phospholipids of brain

slices are labelled, not only from inorganic ^{32}P and [*carboxy*–^{14}C] acetate, but also from ^{-14}C-labelled glycerol, choline, ethanolamine and serine, although brain is less active in this regard than many other tissues (FRIES *et al.*, 1942; STRICKLAND, 1954; PRITCHARD, 1956; ROSSITER, 1957; ROSSITER *et al.*, 1958). These findings, together with the fact that both brain (ROSSITER, MCMURRAY and STRICKLAND, 1957*b*; MCMURRAY, STRICKLAND, BERRY and ROSSITER, 1957) and peripheral nerve (ROSSITER, MCLEOD and STRICKLAND, 1957*a*) contain many of the enzymes necessary for the synthesis of phospholipids, may be put forward as evidence for the view that in brain and peripheral nerve the phospholipids are formed *in situ* from smaller molecules.

Cholesterol. The labelling of cholesterol from [*carboxy*–^{14}C] acetate in tissue slices is well known. However, SRERE, CHAIKOFF, TREITMAN and BURNSTEIN (1949) reported that, except for slices from very young animals, brain tissue was inactive. VAN BRUGGEN, HUTCHENS, CLAYCOMB and WEST (1953) found the same lack of labelling of brain cholesterol when adult rats were given [*carboxy*–^{14}C] acetate. However, MCMILLAN, DOUGLAS and MORTENSEN (1957) reported that if the [*carboxy*–^{14}C] acetate was injected into the cisterna magna instead of into the peritoneal cavity, much better labelling of brain cholesterol was observed.

Our experiments have indicated that there is a slight, but definite, labelling of the cholesterol of brain slices from adult rats (ROSSITER, 1957; ROSSITER *et al.*, 1958). It is now seen that the same is true for fragments of cat sciatic nerve, although degenerating or regenerating nerves are much more active than intact nerves.

In normal nerves all the cholesterol is in the non-esterified form. However, as the nerve degenerates after section or crush, considerable cholesterol ester appears (JOHNSON *et al.*, 1949; BURT *et al.*, 1950). In one experiment with a 32-day nerve, the esterified as well as the non-esterified cholesterol was studied. The ester cholesterol was not labelled. GOULD and TAYLOR (1950) reported that the ester cholesterol of liver slices was not labelled from [^{14}C] acetate.

Degenerating nerves. The experiments reported here indicate that for cat sciatic nerve degenerating after section there is an increase in the *in vitro* labelling of both phospholipid and cholesterol when fragments of the nerve are incubated with [*carboxy*–^{14}C] acetate. Previously MAGEE and ROSSITER (1954) showed that there was an increase in the labelling of phospholipid from inorganic ^{32}P under similar conditions.

These changes suggest that degenerating nerves are more amply provided with the metabolic machinery necessary for the formation of myelin lipids. This is borne out by the finding that degenerating nerve contains higher concentrations of certain of the enzymes concerned with the biosynthesis of phospholipids (ROSSITER *et al.*, 1957*a*, *b*; 1958). Since the Schwann cell is responsible for the formation of myelin (GEREN, 1954), it is presumed that this increase in the capability of the nerve to form myelin lipid (as evidenced by the increased ability to label these lipids) is associated with the increase in the number of Schwann cells known to be present at this time. The Schwann cells form myelin when a suitable opportunity occurs. Such an opportunity occurs during the subsequent regeneration of a nerve that has degenerated as the result of a crushing injury. New axon tips grow outwards from the central stump. The Schwann cells provide these axon tips with successive layers of myelin, i.e. as a result of the activity of the Schwann cells, the nerve becomes remyelinated.

The observation that there was an increase in the labelling of phospholipid and cholesterol in degenerating nerves also is of interest for another reason. FELDBERG (1943) and BANISTER and SCRASE (1950) found that the formation of acetylcholine from choline, acetate, coenzyme A (CoA) and adenosine triphosphate (ATP) decreased rapidly in the superior cervical sympathetic ganglion of the cat after denervation. NACHMANSOHN, JOHN and BERMAN (1946) reported similar findings for the sciatic nerve of the rabbit. It is now known that the first stage in the formation of acetylcholine from choline, acetate, CoA and ATP is the activation of acetate with the formation of acetyl–CoA. Since a similar activation of [^{14}C] acetate is necessary for the labelling of both phospholipid and cholesterol from [^{14}C] acetate, it may be concluded that the poor formation of acetylcholine in degenerating nerves is not the result of a decrease in the formation of acetyl–CoA from acetate. Presumably there is a deficiency in the formation of acetylcholine from acetyl–CoA and choline. Direct findings bearing on this conclusion are reported in the following paper (BERRY and ROSSITER, 1958).

Regenerating nerves. In the nerves regenerating after crush the specific radioactivity of the phospholipid, as well as the radioactivity expressed in terms of counts per min per 100 mg nerve, was greater in the regenerating nerves. The same was true for the labelling of the non-esterified cholesterol. MAGEE and ROSSITER (1954) reported a similar increase for the labelling of phospholipid from inorganic ^{32}P.

It is likely that these increases in the labelling of myelin lipids are related to the production of new myelin that is proceeding rapidly at this time.

SUMMARY

(1) The phospholipid and the non-esterified cholesterol of the sciatic nerve of the cat were both labelled when fragments of the nerve were incubated in a Krebs–Ringer bicarbonate medium containing [*carboxy*–^{14}C] acetate.

(2) In nerves degenerating for 96 days after section and incubated under similar conditions, there was a large increase in the specific radioactivity of both the phospholipid and the non-esterified cholesterol. The radioactivity due to the cholesterol per 100 mg nerve was greater than that of the control nerves, but the figures for phospholipid were more equivocal.

(3) In nerves regenerating 96 days after a crushing injury, there was a large increase in both the specific radioactivity and the radioactivity per 100 mg nerve for both phospholipid and non-esterified cholesterol.

(4) The findings are discussed in relation to current knowledge of the chemistry of Wallerian degeneration. It is suggested that the increase in the labelling of the myelin lipids is related to the proliferation of Schwann cells and that this represents an increase in the ability of the nerve to remyelinate, should a suitable opportunity occur.

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CHEMICAL STUDIES OF PERIPHERAL NERVE DURING WALLERIAN DEGENERATION—VIII

ACETIC THIOKINASE AND CHOLINE ACETYLASE

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THE formation of acetylcholine from choline, acetate, coenzyme A (CoA) and adenosine triphosphate (ATP), or from choline and acetyl–CoA, was measured in extracts of acetone-dried powders prepared from the intact sciatic nerve of the cat and from nerves degenerating after section. In addition, observations were made on the formation of acetyl–CoA from acetate, CoA and ATP. It was found that the formation of acetylcholine either from acetate, CoA and ATP, or from acetyl–CoA, was abolished in sectioned nerve, whereas the formation of acetyl–CoA from acetate was increased.

It is now believed that there are two stages in the synthesis of acetylcholine. The first is the production of acetyl–CoA from CoA and some suitable acetyl-donor. If acetate is the acetyl-donor, the CoA and the acetate react with ATP to form acetyl–CoA, adenosine–5′–monophosphate (AMP) and pyrophosphate (PP) according to the following overall equation: (BEINERT, GREEN, HELE, HIFT, VON KORFF and RAMAKRISHNAN, 1953; HELE, 1954):

$$\text{ATP} + \text{acetate} + \text{CoA} \leftrightharpoons \text{AMP} + \text{PP} + \text{acetyl–CoA} \qquad (1)$$

The mechanism of this reaction, which is catalysed by the enzyme acetic thiokinase, has been the subject of several recent studies (BERG, 1956; WHITEHOUSE, MOESKI and GURIN, 1957).

The second stage in the formation of acetylcholine is the acetylation of choline with acetyl–CoA. KORKES, DEL CAMPILLO, KOREY, STERN, NACHMANSOHN and OCHOA (1952) demonstrated that a number of reactions in which acetyl–CoA is produced could be coupled with the choline acetylase system. Furthermore, acetyl–CoA could be used as a substrate in the enzymic acetylation of choline according to the following reaction:

$$\text{acetyl–CoA} + \text{choline} \leftrightharpoons \text{CoA} + \text{acetylcholine} \qquad (2)$$

The enzyme catalysing this reaction is now termed choline acetylase.

FELDBERG (1943) and BANISTER and SCRASE (1950) reported that the formation of acetylcholine from choline, acetate, CoA and ATP declined rapidly in the superior cervical sympathetic ganglion of the cat after denervation and NACHMANSOHN, JOHN and BERMAN (1946) reported similar findings for the sciatic nerve of the rabbit. From these studies it cannot be determined which of the two stages in the formation of acetylcholine is affected.

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In the previous paper KLINE, MAGEE, PRITCHARD and ROSSITER (1958) reported that the *in vitro* incorporation of [*carboxy*-^{14}C] acetate into the cholesterol and phospholipid of cat sciatic nerve was increased in nerves degenerating after section. Since the labelled acetate is converted into acetyl–CoA before it can be incorporated into cholesterol or phospholipid, the above findings suggest that the reduction in the formation of acetylcholine in degenerating nerve is the result of a decrease in the activity of choline acetylase, rather than a decrease in the activity of acetic thiokinase. The experiments reported in this paper were designed to test this possibility.

Recently HEBB and WAITES (1956), using a liver enzyme system to provide acetyl–CoA, showed that choline acetylase activity was almost completely abolished in the denervated cervical sympathetic nerves and superior cervical ganglia of sheep.

METHODS

The right sciatic nerve of a number of cats was sectioned as described by KLINE *et al.* (1958). After 8, 16, 32, 96, or 632 days the animals were killed and the segment of nerve distal to the site of operation was removed. At the same time, a similar length of intact left sciatic nerve was removed to serve as a control. Each nerve was cleaned of adherent fatty and connective tissue; the epineurium was stripped off and the nerve was rapidly weighed. It was then cut into small pieces and transferred to a mortar where it was frozen with liquid nitrogen and ground to a fine powder. The powder was triturated with 50–100 ml acetone, chilled with dry ice and decanted into 1000 ml acetone-dry ice. After filtration, the caked residue was washed with 600 ml acetone-dry ice and dried in a vacuum desiccator. The dry powder was stored at −15°. The nerves from three cats were pooled for each acetone-dried powder preparation.

Extraction of enzyme. The acetone-dried powders (50 mg) were homogenized with 1 ml 0·007 M–K_2HPO_4 in 0·05 M–KCl (pH 7·0–7·2) in a chilled Potter–Elvehjem homogenizer and centrifuged at 2000 rev/min (1200 × g) for 5 min at 0°. The final volume of the supernatant fluid was adjusted to contain 50 mg powder per ml extract.

Incubation. The enzyme extract (0·3 ml, equivalent to 15 mg acetone-dried powder) was incubated for 90 min at 37° in a medium containing $MgCl_2$ 18·6 μmoles; $CaCl_2$, 14·2 μmoles; KCl, 364 μmoles; K_2HPO_4 (pH 7·0), 21·4 μmoles; KF, 402·5 μmoles; tetraethylpyrophosphate (TEPP)* 18·2 μmoles; reduced glutathione, 3·2 μmoles; reduced CoA, 2·6 μmoles. The total volume was 4·5 ml. The following were present in some flasks as indicated in the tables: choline chloride, 92·1 μmoles; ATP, 50·4 μmoles; potassium acetate, 301 μmoles; neutralized hydroxylamine hydrochloride, 500 μmoles.

Acetyl–CoA was prepared according to WILSON (1952), purified as described by SANADI and LITTLEFIELD (1953), and repurified by paper chromatography as described by STADTMAN (1952). In some experiments acetyl–CoA (0·267 μmoles) was added to the reaction mixture in place of ATP, acetate, and CoA. Control samples contained heat-treated enzyme.

Acetone-dried powders were prepared from rat-brain as described by MUNTZ and HURWITZ (1951). Incubations were carried out as described for cat nerve. In some experiments cytidine triphosphate (CTP), 33·4 μmoles; choline chloride, 9·2 μmoles; ATP, 25·2 μmoles; phosphorylcholine, 9·2 μmoles were added as indicated. Phosphorylcholine was prepared by Dr. K. P. STRICKLAND according to the method of RILEY (1944).

Analyses. Acetylcholine was determined by the method of HESTRIN (1949). Acetyl–CoA was measured by the method of LIPMANN and TUTTLE (1945*a*, *b*) observing the precautions recommended by BEINERT *et al.* (1953). Sulphydryl groups were determined by the method of GRUNERT and PHILLIPS (1951) standardized against reduced glutathione and by iodometric titration according to WOODWARD and FRY (1932).

Spectrophotometric studies. The formation of acetyl–CoA from ATP, CoA, and acetate was measured as the increase in optical density at 232 mμ in the Beckman spectrophotometer according to the methods of STADTMAN (1953) and BEINERT *et al.* (1953). These assays were also carried out in the Warren Spectracord against a blank containing heat-treated enzyme. The system contained K_2ATP,

* Generously provided by Science Service Laboratories, Department of Agriculture, London, Canada.

TABLE 1.—EFFECT OF NERVE SECTION ON THE ACTIVATION OF ACETATE AND THE FORMATION OF ACETYLCHOLINE IN EXTRACTS OF ACETONE-DRIED POWDERS FROM THE SCIATIC NERVE OF THE CAT

Enzyme incubated at 37° for 90 min. Medium contained: $MgCl_2$, 18·6 μmoles; $CaCl_2$, 14·2 μmoles; KCl, 364 μmoles; K_2HPO_4 (pH 7·0), 21·4 μmoles; KF, 402·5 μmoles; TEPP, 18·2 μmoles; reduced glutathione, 3·2 μmoles; CoA, 2·6 μmoles; K_2ATP, 50·4 μmoles; potassium acetate, 301 μmoles; 0·3 ml enzyme extract (equivalent to 15 mg acetone-dried powder). Total volume, 4·5 ml.

Days after section	μmoles/g powder per 90 min*				
	8	16	32	96	632§
Δ*Acetylcholine*†‡					
Intact control	+18·4 ± 2·4	+22·7 ± 3·6	+16·4 ± 3·7	+16·7 ± 4·6	+18·2 ± 5·6
Sectioned	0	0	0	0	0
Δ*Acetyl–CoA*‖					
Intact control	+17·0 ± 1·3	+18·4 ± 2·3	+22·9 ± 2·1	+18·3 ± 3·0	+19·7 ± 0·7
Sectioned	+16·7 ± 1·3	+17·3 ± 2·0	+43·2 ± 4·9	+83·2 ± 2·5	+105·9 ± 4·4
D/N × 100¶	98%	94%	189%	455%	537%
Δ*Sulphydryl*‖					
Intact control	−19·9 ± 1·1	−19·8 ± 3·0	−23·2 ± 1·2	−16·3 ± 0·6	−19·0 ± 0·0
Sectioned	−19·3 ± 2·0	−17·8 ± 1·8	−41·7 ± 2·6	−81·0 ± 2·2	−103·7 ± 0·2
D/N × 100	97%	90%	180%	497%	546%

* Mean ± S.E.M. Each value represents the mean of nine experiments with duplicate samples.
† Medium contained choline chloride, 92·1 μmoles.
‡ Values represent the difference between samples incubated with and without choline.
§ Each value represents the mean of two experiments with duplicate samples.
‖ Medium contained neutralized hydroxylamine hydrochloride, 500 μmoles.
¶ $\frac{\text{values of sectioned}}{\text{values of control}} \times 100$.

TABLE 2.—EFFECT OF NERVE SECTION ON THE UTILIZATION OF ACETYL–CoA FOR ACETYLCHOLINE SYNTHESIS IN EXTRACTS OF ACETONE-DRIED POWDERS FROM THE SCIATIC NERVE OF THE CAT

Enzyme incubated at 37° for 90 min. Medium contained: $MgCl_2$, 18·6 μmoles; $CaCl_2$, 14·2 μmoles; KCl, 364 μmoles; K_2HPO_4 (pH 7·0), 21·4 μmoles; KF, 402·5 μmoles; TEPP, 18·2 μmoles; reduced glutathione, 3·2 μmoles; acetyl–CoA, 0·267 μmoles; choline chloride, 92·1 μmoles; 0·3 ml enzyme extract (equivalent to 15 mg acetone-dried powder). Total volume, 4·5 ml.

Days after section	μmoles/g powder per 90 min*		
	16	32	96
ΔAcetylcholine			
Intact control	+16·4 ± 0·7	+15·3 ± 0·4	+16·5 ± 0·7
Sectioned	0	0	0

* Mean ± S.E.M. Each value represents the mean of two experiments with duplicate samples.

5 μmoles; reduced CoA, 0·26 μmoles; potassium acetate, 30 μmoles; reduced glutathione, 0·3 μmoles; $MgCl_2$, 15·4 μmoles; $CaCl_2$, 11·8 μmoles; K_2HPO_4 (pH 7·0), 17·7 μmoles; KCl, 184 μmoles; KF 204 μmoles; enzyme extract 0·03 ml. The total volume was 3·0 ml and the system was comparable to that used by STADTMAN (1953) and BEINERT *et al.* (1953).

TABLE 3.—EFFECT OF PHOSPHORYLCHOLINE AND CTP ON THE SYNTHESIS OF ACETYLCHOLINE IN EXTRACTS OF ACETONE-DRIED POWDERS OF RAT BRAIN

Enzyme incubated for 90 min at 37°. Medium contained $MgCl_2$, 18·6 μmoles; $CaCl_2$, 14·2 μmoles; KCl, 364 μmoles; K_2HPO_4 pH 7·0, 21·4 μmoles; KF, 402·5 μmoles; TEPP, 18·2 μmoles; reduced glutathione, 3·2 μmoles; enzyme extract (0·3 ml, equivalent to 15 mg acetone-dried powder). The following were added as indicated: Na_2CTP, 33·4 μmoles; choline chloride; 9·2 μmoles; reduced CoA, 2·6 μmoles; potassium acetate, 301 μmoles; acetyl–CoA, 0·267 μmoles. Total volume, 4·5 ml

Choline	Acetate	CoA	ATP	Acetyl –CoA	CTP	Phosphoryl-choline	μmoles acetylcholine/g powder per 90 min*
+	+	+	+	−	−	−	14·1 ± 2·4
+	+	+	+	−	+	−	12·7 ± 2·0
+	+	+	+	−	−	+	17·8 ± 2·0
+	−	−	−	+	−	−	17·0 ± 1·0
+	−	−	−	+	+	−	17·0 ± 2·5
+	−	−	−	+	−	+	17·1 ± 2·5

* Mean ± S.E.M. Each value represents the mean of four experiments with duplicate samples.

RESULTS

Table 1 shows that there was no detectable formation of acetylcholine from choline, acetate, CoA, and ATP in cat sciatic nerve as early as 8 days after section, whereas the formation of acetyl–CoA from acetate, CoA, and ATP was greatly increased at 32 days and continued to increase up to 632 days after section. The table also shows that there is a reasonable stoichiometric correlation between the formation of acetyl–CoA and the disappearance of free sulphydryl groups in both intact nerves and degenerating nerves at all time intervals after the operation. The failure of the degenerating nerves to synthesize acetylcholine at a time when the activity of acetic thiokinase is normal or increased strongly suggests that the decreased synthesis is the result of an absence of choline acetylase activity.

The above conclusion is confirmed by the experiments reported in Table 2, which demonstrate directly the inability of sectioned nerve to utilize synthetic acetyl–CoA for the formation of acetylcholine.

The increased thiokinase activity of degenerating nerves also was studied spectrophotometrically. Figures 1 and 2 present the results of two typical experiments in which the formation of acetyl–CoA from acetate, CoA and ATP was followed by observing the increase in optical density at 232 mμ. In enzyme preparations from nerves degenerating 32 or 96 days after section the initial rate of formation of acetyl–CoA was greater than that in similar enzyme preparations from intact control nerves. The final steady-state concentrations of acetyl–CoA also were increased 32 and 96 days after section.

Table 3 shows that the addition of CTP had no significant effect on the synthesis of acetylcholine either from choline, acetate and ATP, or from choline and acetyl–CoA,

in extracts of acetone-dried powder of rat brain. The small differences in the mean values for the synthesis of acetylcholine were not significant statistically.

Table 3 also shows that there was a slight increase in the mean value for the synthesis of acetylcholine from acetate and ATP when phosphorylcholine was added to the system, not observed when acetyl–CoA was the acetyl-donor.

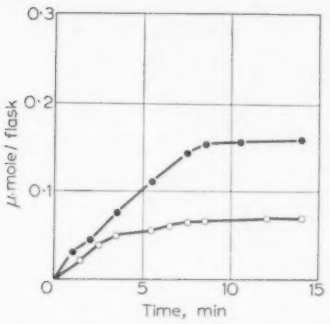

FIG. 1.—Spectrophotometric estimation of acetic thiokinase activity in extracts of acetone-dried powders from an intact (—○—○—) and a degenerating (—⊗—⊗—) sciatic nerve from a cat. Time after nerve section, 32 days. Ordinate, μmoles acetyl–CoA per flask.

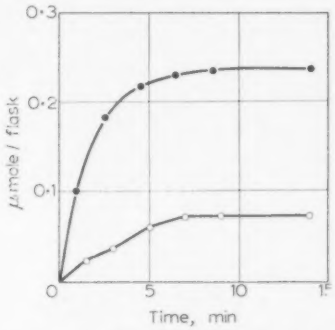

FIG. 2.—Spectrophotometric estimation of acetic thiokinase activity in extracts of acetone-dried powders from an intact (—○—○—) and a degenerating (—⊗—⊗—) sciatic nerve from a cat. Time after nerve section, 96 days. Ordinate, μmoles acetyl–CoA per flask.

DISCUSSION

These experiments confirm the results of previous workers that there is a decrease in the formation of acetylcholine in extracts of acetone-dried powders prepared from degenerating nerves. They further show that this decrease is the result of a loss in the activity of the enzyme that catalyses the acetylation of choline from acetyl–CoA and that it is not the result of a decrease in the formation of acetyl–CoA from acetate. Such a conclusion is in accord with the recent report of HEBB and WAITES (1956).

The increase in the activity of acetic thiokinase in peripheral nerve degenerating 32 days after section is consistent with the findings reported in the previous paper that there was an increase in the incorporation of [*carboxy*-^{14}C] acetate into the cholesterol and phospholipid of degenerating nerve (KLINE *et al.*, 1958). It is known that [^{14}C] acetate is activated to form [^{14}C] acetyl–CoA before the radioactivity is incorporated into cholesterol or phospholipid, so that any increase in acetic thiokinase activity would facilitate the labelling of these lipids. It is possible that the increase in the activity of acetic thiokinase is the result of the proliferation of Schwann cells that occurs after nerve section.

KENNEDY and WEISS (1956) recently showed that during the incorporation of choline into lecithin, phosphorylcholine is activated by CTP to form the intermediate cytidine diphosphate choline. In the present experiments, the addition of CTP did not increase the formation of acetylcholine, either from ATP, acetate and CoA, or from acetyl–CoA, suggesting that this nucleotide does not function as a cofactor in acetylcholine formation.

The addition of phosphorylcholine had no effect on the formation of acetylcholine from acetyl–CoA. However, when the precursors were acetate, CoA and ATP, the

mean figure for the formation of acetylcholine was less in the presence of phosphorylcholine ($P = 0{\cdot}1 - 0{\cdot}05$), although this decrease was of doubtful statistical significance. In the following paper it is suggested that the addition of phosphorylcholine to such a system might spare ATP for acetate activation and the subsequent synthesis of acetylcholine (BERRY, MCPHERSON and ROSSITER, 1958).

SUMMARY

(1) The formation of acetylcholine from choline, acetate, CoA and ATP was not detected in extracts of acetone-dried powders prepared from cat sciatic nerves 8, 16, 32, 96 and 632 days after section.

(2) There was no significant change in the formation of acetyl–CoA from acetate, CoA and ATP in extracts of acetone-dried powders prepared from cat sciatic nerves 8 and 16 days after nerve section, whereas there was a progressive increase after 32 days.

(3) These findings indicate that in enzyme preparations from nerves degenerating after section the decrease in the formation of acetylcholine is the result of a loss of choline acetylase activity and is not the result of a decrease in the activity of acetic thiokinase. This conclusion is confirmed by the finding that there was also a failure of such enzyme preparations to form acetylcholine from choline and acetyl–CoA.

(4) The addition of CTP, or phosphorylcholine, had no significant effect on the synthesis of acetylcholine in extracts of acetone-dried powders prepared from rat brain.

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CHEMICAL STUDIES OF PERIPHERAL NERVE DURING WALLERIAN DEGENERATION—IX

CHOLINE KINASE

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CHOLINE kinase activity was estimated in extracts of acetone-dried powders prepared from normal cat sciatic nerve and nerves degenerating after section. The activity of this enzyme was unchanged 16 days after section, but was greatly decreased by 32 days. In addition, it was found that added phosphorylcholine inhibited the choline kinase activity in similar enzyme preparations obtained from rat brain.

Previously it was shown that the concentration of phospholipid decreased rapidly in sectioned nerves during the period 8 to 32 days after operation (JOHNSON, MCNABB and ROSSITER, 1949; LOGAN, MANNELL and ROSSITER, 1952), whereas the *in vitro* incorporation of inorganic ^{32}P into the phospholipid of fragments of cat sciatic nerve increased during this same period (MAGEE and ROSSITER, 1952). Recently MAGEE, BERRY, MAGEE and ROSSITER (1958) have shown that this increase in the incorporation of inorganic ^{32}P into phospholipid could not be correlated with the changes in the concentration or the specific radioactivity of adenosine triphosphate (ATP). These observations were in contrast to the effects of inhibitors on the ATP of water dispersions of rat brain (BERRY and MCMURRAY, 1957) or rat brain mitochondria prepared from sucrose homogenates (MCMURRAY, BERRY and ROSSITER, 1957*a*), where changes in the incorporation of inorganic ^{32}P into phospholipid were accompanied by changes in concentration or specific radioactivity of ATP, or both.

It was considered that the changes in the concentration and specific radioactivity of phospholipid in degenerating nerve possibly might be explained on the basis of changes in phosphorylation reactions that occur after the stage of the incorporation of inorganic ^{32}P into ATP. WITTENBERG and KORNBERG (1953) showed that extracts of acetone-dried powders of brain and other tissues contained an enzyme, which they called choline phosphokinase, that catalysed the phosphorylation of choline by ATP, with the formation of phosphorylcholine and adenosine diphosphate (ADP). This represents the step that follows the formation of ^{32}P-labelled ATP in the sequence of reactions proposed by KENNEDY (1956) for the formation of ^{32}P-labelled lecithin. MCMURRAY, STRICKLAND, BERRY and ROSSITER (1957*b*) showed that similar reactions occur in tissue from the nervous system.

In the present experiments, choline kinase activity was determined in normal nerves and in nerves degenerating after section.

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METHODS

The right sciatic nerve of a number of cats was sectioned as described by KLINE, MAGEE, PRITCHARD and ROSSITER (1958). After 16 or 32 days the animals were killed, and the segment of the nerve distal to the site of the operation was removed. At the same time a similar length of the intact left sciatic nerve was removed to serve as a control. Each nerve was cleaned of adherent fatty and connective tissue; the epineurium was stripped off and the nerve was rapidly weighed. Acetone-dried powders of these nerves were then prepared and stored as described by BERRY and ROSSITER (1958). The nerves from three cats were pooled for each acetone-dried powder preparation.

Extraction of enzyme. Acetone-dried powder (80 mg) was homogenized with a small volume 0·04 M-glycylglycine buffer (pH 8·5) in a chilled Potter–Elvehjem homogenizer and centrifuged at 2000 rev/min (1200 × g) for 10 min at 0°. The final volume of the supernatant fluid was adjusted to 0·8 ml, equivalent to 100 mg powder per ml.

Incubation. Samples were incubated with shaking in stoppered test tubes at 37° for 90 min. Each tube contained 0·2 or 0·3 ml enzyme extract (equivalent to 20 mg acetone-dried powder), glycylglycine (pH 8·5), 40 μmoles; $MgCl_2$, 12 μmoles; reduced glutathione, 4·2 μmoles; ATP, 9 μmoles; choline chloride, 2 μmoles, in a total volume of 1·0 ml. Zero-time tubes contained initially 0·2 ml ice-cold 60% (w/v) trichloroacetic acid (TCA) to which was added the enzyme and incubation medium. After 90 min the reaction in the other tubes was stopped by addition of 0·2 ml ice-cold 60% TCA and the samples were filtered in the cold.

Chromatography. The filtrates, without preliminary desalting which was found to be unnecessary, were applied to filter paper.

For the separation of phosphorylcholine, duplicate 300 or 500 μl samples were applied to Whatman No. 1 filter paper and treated with the phenol–water solvent as described by ROUSER, BERRY, MARINETTI and STOTZ (1953). Authentic phosphorylcholine had an R_P value of 0·87, as reported by OLLEY and DAWSON (1956). Adenosine phosphates were also separated in this solvent system. In addition, the adenosine phosphates and inorganic phosphate were separated by the method of EGGLESTON and HEMS (1952). Duplicate 50 μl samples were applied to Whatman No. 4 filter paper and treated as described by BERRY and MCMURRAY (1957). Phosphorylcholine was also separated from the adenosine phosphates and inorganic P in this system.

Standard spots containing inorganic P, phosphorylcholine, ATP, ADP and adenosine-5′-monophosphate (AMP), in quantities equivalent to 15–20 μg P/compound, were run with each chromatogram. Spots were located by examination with a 'Mineralight' short-wave ultraviolet lamp and by the procedure of HANES and ISHERWOOD (1948). The material was eluted from the areas corresponding to inorganic P, phosphorylcholine, ATP, and ADP with approximately 3 ml distilled water and ashed with 1 ml 60% (w/v) perchloric acid. Phosphorus was estimated by the procedure of ERNSTER, ZETTERSTRÖM and LINDBERG (1950).

Rat brain acetone-dried powders

Acetone-dried powders of rat brain were prepared as described for cat nerve, or as described by MUNTZ and HURWITZ (1951). The acetone-dried brain powder (320 mg) was homogenized with 2 ml 0·05 M-KCl in 0·007 M-K_2HPO_4 (pH 7·0) in a chilled Potter–Elvehjem homogenizer and centrifuged at 0° for 10 min at 2000 rev/min (1200 × g). The final volume of the supernatant fluid was adjusted to 1·6 ml.

In each experiment 0·1 ml enzyme extract (equivalent to 20 mg acetone-dried powder) was incubated in the buffered medium of BERRY and STOTZ (1956). The total volume was 1 ml. Incubation, chromatography, and analyses were carried out as described for cat nerve.

Reagents. ATP was the Pabst crystalline disodium salt, free of non-adenine nucleotides. Phosphorylcholine was prepared by Dr. K. P. STRICKLAND according to the method of RILEY (1944).

RESULTS

Choline kinase activity in intact and degenerating nerve

Table 1 shows the formation of phosphorylcholine and the changes that occur in the concentrations of ATP, ADP and inorganic P in extracts of acetone-dried powders prepared from intact and degenerating cat sciatic nerves. In enzyme preparations

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TABLE I.—EFFECT OF NERVE SECTION ON THE CHOLINE KINASE ACTIVITY IN EXTRACTS OF ACETONE-DRIED POWDERS FROM THE SCIATIC NERVE OF THE CAT

Enzyme incubated at 37° for 90 min. Medium contained: glycylglycine (pH 8·5), 40 μmoles; $MgCl_2$, 12 μmoles; reduced glutathione, 4·2 μmoles; ATP, 9 μmoles; choline chloride, 2 μmoles; 0·2 or 0·3 ml enzyme extract (20 mg acetone-dried powder). Total volume, 1·0 ml.

	μmoles compound/g powder per 90 min*				
	Δ Phosphorylcholine	Δ ATP	Δ ADP	Δ Inorganic P	Δ Phosphorylcholine + Δ inorganic P
Intact control	+73·5 ± 11·0	−84·5 ± 8·0	+86·5 ± 10·0	+14·5 ± 0·0	+88·0
16-day sectioned	+81·2 ± 9·3	−110·5 ± 22·5	+114·7 ± 21·8	+19·7 ± 3·3	+100·9
P†	>0·6	>0·4	>0·3	>0·3	
D/N‡ × 100	110%	131%	132%	136%	
Intact control	+85·7 ± 2·2	−106·7 ± 2·2	+105·5 ± 4·5	+23·0 ± 1·5	+108·7
32-day sectioned	+32·0 ± 1·5	−58·7 ± 3·3	+ 60·7 ± 6·3	+27·5 ± 1·0	+59·5
P	<0·001	<0·001	<0·01	>0·05	
D/N × 100	37%	55%	58%	117%	

* Mean ± S.E.M. Each value is the mean of 8 observations.
† P value as determined by Fisher's t-test.
‡ $\frac{\text{values for the sectioned nerve}}{\text{values for control nerve.}} \times 100$

from nerves studied 16 days after section the figures for the formation of phosphorylcholine, the disappearance of ATP, and the appearance of ADP were not significantly different from the corresponding values for intact nerves from the opposite side. There was thus no significant change in choline kinase activity 16 days after operation.

However, by 32 days there was a 60–70 per cent decrease in the formation of phosphorylcholine. This was accompanied by a 40–50 per cent decrease in the disappearance of ATP and in the appearance of ADP. These results indicate that the choline kinase activity was significantly decreased in nerves degenerating 32 days after section. There was no significant difference in the amount of inorganic P that was liberated. This finding suggests that the decreases in the consumption of ATP and in the production of phosphorylcholine were not due to changes in the activity of either a phosphatase or an ATPase.

It may also be seen from Table 1 that, in each instance, the disappearance of ATP is balanced by the appearance of ADP. The disappearance of ATP also is balanced by the sum of the figures for the appearance of phosphorylcholine and inorganic P.

The lack of significant change in choline kinase activity in the 16-day sectioned nerves and the decrease at 32 days were both evident whether the results were expressed in terms of unit weight of acetone-dried powder, or in terms of unit length of fresh nerve.

Inhibition of rat brain choline kinase by phosphorylcholine

Table 2 shows that in extracts of acetone-dried powders of rat brain the amount of phosphorylcholine formed during the experimental period was less when high concentrations of phosphorylcholine were added to the medium. When the concentration of phosphorylcholine was 5×10^{-3} M, the formation of phosphorylcholine was inhibited by 50 per cent. The changes in the formation of phosphorylcholine were accompanied by corresponding, but not identical, decreases in the consumption of ATP and the production of ADP, with no significant change in the formation of inorganic phosphate.

The results indicate that there is a significant inhibition of choline kinase activity when phosphorylcholine is present in concentrations greater than $2{\cdot}5 \times 10^{-3}$ M.

In the experiments with rat brain reported in Table 2, like the experiments with cat peripheral nerve reported in Table 1, the consumption of ATP is balanced by the production of ADP and also by the sum of the increases in concentrations of phosphorylcholine and inorganic phosphate.

DISCUSSION

The experiments show that there was no change in the activity of choline kinase in enzyme preparations from cat sciatic nerves degenerating 16 days after section, but that by 32 days the activity of the enzyme was considerably decreased.

During the course of the incubation small amounts of inorganic P were liberated. This indicates that the enzyme preparation may have possessed some alkaline phosphomonoesterase activity and, possibly, some ATPase activity. STRICKLAND, THOMPSON and WEBSTER (1956) showed that inorganic P was liberated from phosphorylcholine in extracts of acetone-dried powders of brains of a number of different species. However, the small amount of inorganic P liberated, compared with the amounts of phosphorylcholine formed and ATP broken down, indicates that the

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TABLE 2—EFFECT OF ADDED PHOSPHORYLCHOLINE ON CHOLINE KINASE ACTIVITY IN EXTRACTS OF ACETONE-DRIED POWDERS FROM RAT BRAIN

Enzyme incubated at 37° for 90 min. Medium contained: K_2HPO_4 (pH 7·0), 3·9 μmoles; $MgCl_2$, 1·4 μmoles; ATP, 9 μmoles; $CaCl_2$, 1·2 μmoles; KCl, 62 μmoles; KF, 31 μmoles; choline chloride, 2 μmoles; glutathione, 4·2 μmoles; phosphorylcholine as indicated; 0·1 ml enzyme extract (20 mg acetone powder). Total volume, 1·0 ml.

Phosphoryl-choline concentration	Δ Phosphorylcholine (μmoles compound per flask per 90 min)	(% control)	Δ ATP (μmoles compound per flask per 90 min)	(% control)	Δ ADP (μmoles compound per flask per 90 min)	(% control)	Δ Inorganic P (μmoles compound per flask per 90 min)	(% control)	Δ Phosphoryl-choline + Δ inorganic P
0 M	+1·15	100	−1·91	100	+1·99	100	+0·76	100	+1·91
5×10^{-4} M	+1·22	106	−2·05	107	+2·09	104	+0·84	110	+2·06
10^{-3} M	+1·15	100	−2·10	100	+2·01	101	+0·76	100	+1·91
$2·5 \times 10^{-3}$ M	+0·80	70	−1·66	87	+1·66	88	+0·82	108	+1·62
5×10^{-3} M	+0·54	47	−1·24	65	+1·32	75	+0·70	92	+1·24

activity of alkaline phosphomonoesterase and ATPase could not have been great in our experiments and could not have been responsible for the differences between the intact and degenerating nerves.

The decrease in the concentration of the phosphorylcholine-containing lipids, phosphatidylcholine and sphingomyelin, reported by JOHNSON *et al.* (1949) for degenerating nerve, possibly could be related to a decrease in the activity of choline kinase. However, such a decrease in choline kinase activity offers no explanation for the increase in the net incorporation of inorganic ^{32}P into total phospholipid (MAGEE and ROSSITER, 1954) or into phosphatidylcholine (MAGEE *et al.*, 1958) that occurs in nerves degenerating after section.

It is possible that the changes in the labelling of phospholipid that accompany degeneration are more adequately explained by changes in the activities of enzymes at a stage in the synthesis of phospholipid subsequent to the choline kinase reaction. This is suggested by the recent findings of ROSSITER, MCLEOD and STRICKLAND (1957) that homogenates prepared from degenerating rat sciatic nerves are able more readily to incorporate the [^{32}P]phosphorylcholine of [^{32}P]cytidine diphosphate choline into lipid than are similar homogenates prepared from intact nerves.

The presence of phosphorylcholine in concentrations exceeding $2{\cdot}5 \times 10^{-3}$ M inhibits the formation of phosphorylcholine in extracts of acetone-dried powders from rat brain. RAMASARMA and WETTER (1957) observed a similar inhibition by phosphorylcholine of choline kinase prepared from aqueous extracts of Polish rapeseed. Previously, BERRY and STOTZ (1956) had reported an increase in the formation of acetylcholine, an ATP-requiring reaction, when phosphorylcholine was added to extracts of acetone-dried powders of rat brain. It was tentatively suggested that the choline kinase reaction of WITTENBERG and KORNBERG (1953) was reversible and that the added phosphorylcholine might supply the ATP necessary for the synthesis of acetylcholine. However, MCMURRAY *et al.* (1957*b*) failed to detect any exchange of label between [^{32}P] phosphorylcholine and ATP, indicating that such an explanation is unlikely.

The highest concentration of phosphorylcholine used in the present study (5×10^{-3} M), which caused a 50 per cent inhibition of the formation of phosphorylcholine, caused a 35 per cent inhibition in the consumption of ATP. A similar concentration of phosphorylcholine was found to cause a 40 per cent increase in the formation of acetylcholine in the brain choline acetylase system studied by BERRY and STOTZ (1956). Presumably, under these conditions both acetylcholine and phosphorylcholine are formed simultaneously in the presence of ATP, choline, acetate and CoA, as indicated by the work of KOMETIANI (1952). However, when sufficient phosphorylcholine is added, or phosphorylcholine is allowed to accumulate, the choline kinase reaction is inhibited and ATP is spared for the activation of acetate and the synthesis of acetylcholine.

SUMMARY

(1) The values for the appearance of phosphorylcholine, ADP and inorganic phosphate, and for the disappearance of ATP in extracts of acetone-dried powders from cat sciatic nerves degenerating 16 days after section were not significantly different from corresponding values for intact control nerves.

(2) In nerves studied 32 days after section, there was a 60–70 per cent decrease in

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the formation of phosphorylcholine and a 40–50 per cent decrease in both the consumption of ATP and the appearance of ADP, indicating a decrease in the activity of choline kinase.

(3) The significance of these findings is discussed in relation to previous observations on the concentration and the ^{32}P-labelling of phospholipid in normal and degenerating nerve.

(4) Added phosphorylcholine, at concentrations of $2{\cdot}5 \times 10^{-3}$ M or greater, inhibited the formation of phosphorylcholine in extracts from rat-brain acetone-dried powders.

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METABOLISM OF GLUCOSE AND OXYGEN IN A MAMMALIAN SYMPATHETIC GANGLION AT REDUCED TEMPERATURE AND VARIED pH

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THIS paper is concerned with substrate utilization in neuronal metabolism, and particularly with the possibility that a substrate may be oxidized during physiological activity which is different from that oxidized in a neurone at rest. The experiments lead to the conclusion that glucose may be the only substrate oxidized by either active or resting nerve cells under some circumstances, although there are other conditions under which glucose metabolism fails to account for the observed rate of oxygen consumption during activity. Variables influencing this metabolic change include temperature and pH.†

HOLMES, GERARD and SOLOMON concluded in 1930 that carbohydrate metabolism adequately accounted for the rate at which oxygen was consumed at rest in nerve trunks of frogs and rabbits. However, carbohydrate utilization did not accelerate during activity and therefore did not account for the increase in rate of oxygen uptake which occurred when a nerve was stimulated. In these experiments the nerve trunks were suspended in a gas phase and were therefore not provided with an external supply of carbohydrate on which to draw. Moreover the measurements were restricted to changes in the concentration of reducing substances which were only partly identified and to changes in the concentration of lactic acid within the nerve trunks. In contrast to the findings of these investigators, several others who have measured uptake of glucose from a bathing fluid or perfusion solution have all found accelerated consumption of this carbohydrate during electrical stimulation or during activity caused by other means in various nervous tissues. This includes the work of HIRSCHBERG and WINTERSTEIN (1917, 1919) on excised spinal cords and nerves of frogs, MCILWAIN, ANGUIANO and CHESHIRE (1951) on slices of guinea pig brain, GEIGER, MAGNES and DOBKIN (1954) on perfused cat brain, and HOROWICZ and LARRABEE (1958) on sympathetic ganglia excised from rats. However, except for HIRSCHBERG and WINTERSTEIN, who did not measure lactate output, all these investigations supported one conclusion reached by HOLMES and his colleagues: no more carbohydrate was oxidized during activity than at rest, since in each case the increase in glucose taken up was approximately equalled by an increase in lactate put out. Thus endogenous substrates must have been burned by the additional oxygen utilized during activity. A similar conclusion was reached by MULLINS (1953) for frog's nerve, since he found no increase during stimulation in the yield of radioactive CO_2 from glucose randomly labelled with carbon-14. He did, however, obtain evidence for accelerated oxidation of certain amino acids.

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† Preliminary reports have been published by DOLIVO and LARRABEE (1957, 1958)

These lines of evidence, indicating oxidation of substrates other than exogenous glucose during neuronal activity in excised and perfused tissues, appear to conflict with studies of human brain *in situ*, where a balance has been found between glucose and oxygen metabolism after allowance is made for conversion of some of the glucose to lactate and to pyruvate (HIMWICH and HIMWICH, 1946). It should be emphasized that this balance occurs in brain *in situ* despite the unquestionable presence of continual spontaneous neuronal activity. Thus one might conclude that in this case oxidations are limited to glucose, during activity as well as rest. There is, however, a difficulty in interpreting the human data, since it is not known how many of the neurones are normally active and at what frequency these active cells are firing. Thus, even if the metabolism associated with activity utilized a non-carbohydrate substrate, this might be a trivial and undetected fraction of the whole brain metabolism, although quite essential for normal physiological function in those neurones which happen to be in repetitive action. Accordingly the problem of substrate utilization during activity is worthy of further experimental investigation.

The experiments to be described in this paper were conducted on superior cervical ganglia excised from rats. These experiments at reduced temperatures were initiated at a time when we were concerned about possible effects of anoxic cores, which may have been present in some excised ganglion preparations at high temperatures (LARRABEE, 1958). Although appropriate controls finally indicated that anoxia was not a significant factor in the earlier glucose and lactate measurements at 36°C (HOROWICZ and LARRABEE, 1958), the results at reduced temperature are being reported because they have revealed significant effects of changes in temperature and pH. The particular temperature of 23° was selected as about the lowest at which most of the ganglion cells responded to repetitive volleys of preganglionic nerve impulses at a sufficient frequency and over long enough periods of time to permit adequate measurements of the metabolic changes accompanying neuronal activity.

METHODS

Methods were similar to those previously described (LARRABEE, 1958; HOROWICZ and LARRABEE, 1958; LARRABEE, HOROWICZ, STEKIEL and DOLIVO, 1957) except for a change of experimental temperature from 36° to 23–25°. Superior cervical ganglia, together with 10–15 mm lengths of preganglionic nerve and 2–3 mm lengths of postganglionic nerve, were excised from adult hooded rats of both sexes (Lashley strain) with body weights usually between 180 and 220 g. The animals were anaesthetized with urethane (1–1·5 g/kg) administered intraperitoneally.

The bathing solution, prepared under sterile conditions, contained 136 mM-NaCl, 5·6 mM-KCl, 2·22 mM-$CaCl_2$, 1·2 mM-$MgCl_2$, 1·2 mM-NaH_2PO_4, 5·5 mM-glucose, and 16·2 mM-$NaHCO_3$. This solution was equilibrated with 5% CO_2, 95% O_2. The pH was adjusted to various values by addition of NaOH or HCl. In the absence of such addition the pH was about 7·1 at 23°.

At the end of the experiment the preparation was dried to constant weight by placing in an oven at 90° to 100° for at least ½ hr. All metabolic rates reported in this paper are based on dry weight of tissue, which was usually between 200 and 350 μg. Average ratios of wet weight to dry weight, determined by three different observers in this laboratory, were as follows, with numbers of preparations in parentheses: 4·2 (49), 4·3 (29), 4·7 (13).

Glucose uptake and lactate output. The preparation was mounted on a small cluster of platinum electrodes imbedded in glass, which permitted stimulation of the preganglionic nerve and recording from the postganglionic nerve. The height of the postganglionic action potential was measured frequently throughout the course of each experiment. Care was taken to keep the preganglionic stimuli at supramaximal strength. The preparation was dipped into a small quantity of bathing fluid (about 30 μl) for about 12 sec of each minute and was exposed to moist 5% CO_2, 95% O_2 for the remainder of the time. The bathing solution was replaced with fresh solution about ½ hr after

putting the preparation in the apparatus and at intervals varying from ½ to 2 hr thereafter. The samples of solution which had been exposed to the tissue, as well as several control samples, were analysed for glucose and lactate by the methods of PARK and JOHNSON (1949) and BARKER and SUMMERSON (1941) respectively. Rates of glucose uptake and lactate output were calculated from the changes in concentration in the bathing fluid. For further details see HOROWICZ and LARRABEE (1958).

The apparatus used previously was modified by addition of a switch which automatically turned off the stimulating current for about 12 sec each time the preparation was immersed in the bathing fluid. This prevented current from being passed through the fluid. In order to insure that the flow rate of the O_2–CO_2 mixture was sufficient to prevent entry of air, a meter was installed in the gas line and the flow rate was adjusted to about 18 ml/min.

Improvement in reproducibility of the analytical results was achieved by calibration of the test tubes used for cuvettes, not only with respect to optical path length through the solution, but also for the absorption of light by the glass at the wavelengths of the glucose and lactate readings. Each spectrophotometer reading was corrected for both of these factors. Analyses were always made on the day following the experiment, never on the second day as in some of the earlier experiments. After an unfortunate experience with contamination by acetaldehyde, no volatile materials were allowed in the refrigerator where the samples were stored. The concentration of Duponol used in the glucose analyses was reduced from that used by HOROWICZ and LARRABEE (1958) to 1 g/l. in the ferric iron solution, as originally recommended by PARK and JOHNSON (1949). This concentration was sufficient provided the cuvettes were shaken just before insertion into the spectrophotometer.

Another change from previous procedures was to discontinue the use of an autoclave for sterilizing the dissecting instruments, the small glass vessels for the bathing fluid, etc. Instead, this equipment was placed in an oven at 90–100° for 2–3 hr. This was evidently adequate protection against growth of micro-organisms since there was no tendency for the metabolic rates to rise spontaneously during the course of an experiment.

Oxygen uptake. The rate of oxygen uptake was measured under sterile conditions in the continuous-flow respirometer described by LARRABEE (1958). In this instrument, solution which had been equilibrated with 5% CO_2, 95% O_2 flowed slowly at a known rate through a small glass chamber containing the tissue. The fraction of oxygen remaining in the solution leaving the chamber was measured with a polarized platinum electrode. The rate of oxygen uptake could be calculated from the fraction of oxygen removed by the tissue, the flow rate of solution, and the solubility of oxygen in the solution at the temperature of the experiment.

The respirometer contained electrodes for stimulating the preganglionic nerve and for recording from the preganglionic and postganglionic nerves.

RESULTS

A. Rates of Glucose and Oxygen Metabolism at Rest

Results of the first 2 hr of metabolic measurements on a number of resting ganglion preparations are summarized in Table 1. These measurements began 1–1½ hr after excision. The data have been divided into two ranges of pH; the higher range (7·4 to 7·1, column I) includes the normal pH of the blood. In addition to the measured rates of glucose uptake and lactate output, the difference between these two rates is given in the table. This difference indicates the rate at which glucose became available for possible oxidation or other uses after allowing for lactate formation. This will sometimes be referred to in this paper as the 'glucose remainder.' Application of the *t*-test showed that the only statistically significant change caused by alteration of pH was that in rate of lactate production. Reduction of lactate output caused by lowering the pH has also been observed under aerobic conditions in suspensions of rat brain tissue (BIRMINGHAM and ELLIOTT, 1951).

Glucose metabolism is compared with oxygen uptake in Table 2 at both 23° and 36°. Because most measurements of oxygen uptake at the lower temperature were made at a pH of about 7·1, the comparison at this temperature (column I) has been

TABLE 1.—RESTING METABOLISM AT 23° DURING FIRST 2 HR OF MEASUREMENT

	I	II	III	IV
pH	7·45–7·10	7·09–6·45	Difference	*t*-test of difference
A. Glucose uptake	4·62 ± 0·22 (35)	4·15 ± 0·25 (18)	0·47	$P > 0·1$ (not significant)
B. Lactate output	1·37 ± 0·09 (35)	0·88 ± 0·12 (18)	0·49	$P < 0·01$ (significant)
C. Difference between glucose uptake and lactate output	3·25 ± 0·21 (35)	3·27 ± 0·21 (18)	0·02	$P > 0·9$ (not significant)

Mean ± S.E.M. in mg/g dry weight/hr. Number of preparations in parentheses.
Columns III and IV refer to the differences between the average rates in the two pH ranges.

TABLE 2.—COMPARISON OF GLUCOSE AND OXYGEN METABOLISM AT REST AT 23° AND 36°

	I	II
Temperature (°C)	23	36
pH	6·45–7·45	7·3–7·4
mg/g dry weight/hr		
A. Glucose uptake	4·46 ± 0·17 (53)	11·68 ± 0·33 (46)†
B. Lactate output	1·20 ± 0·08 (53)	3·44 ± 0·19 (46)†
C. Difference between glucose uptake and lactate output	3·26 ± 0·16 (53)	8·23 ± 0·41 (46)†
μmoles/g dry weight/hr		
D. Oxygen equivalent of difference	108·5 ± 5·3 (53)	274 ± 14 (46)†
E. Oxygen uptake	110·9 ± 7·8 (10)*	300 ± 9 (23)‡
F. Oxygen excess (E − D)	2·4	26
G. *t*-test of oxygen excess	$P > 0.8$ (not significant)	$P > 0·2$ (not significant)

Mean ± S.E.M. Number of preparations in parentheses.

* Measured at approximately pH 7·1, temperature 23–25°. (LARRABEE, 1958).
† HOROWICZ and LARRABEE (1958).
‡ LARRABEE (1958).

made with the average results of all the glucose and lactate experiments, which were distributed on both sides of pH 7·1. Actually, the method of selecting data with respect to pH is probably of no great importance in this comparison, since there was evidence that neither the glucose remainder nor the rate of oxygen uptake varied significantly with pH in resting ganglion preparations at 23–25°. The finding that rate of oxygen uptake is relatively independent of pH over the range investigated is in agreement with observations of ELLIOTT and BIRMINGHAM (1949) on suspensions of rat brain. They found a broad maximum in respiratory rate with relatively little change from pH 6·5 to pH 7·3.

Results from the present experiments at 23°, listed in column I of Table 2, show

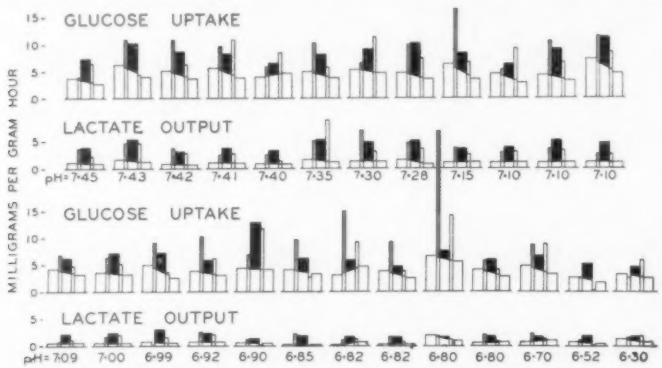

FIG. 1. Rates of glucose uptake and lactate output of twenty-five superior cervical sympathetic ganglia excised from rats. The pH of the bathing fluid is stated below the pair of graphs for each experiment. Each experiment was divided into five periods totalling 7 hr. The preganglionic nerve was stimulated supramaximally at an average frequency of 4·8/sec during the second and third preiods. Resting rates during the second, third and fourth periods are indicated by linear interpolation between the initial and final periods. Increments above these interpolated resting rates may be ascribed to effects of activity. 23°.

that the rate of oxygen consumption was in good agreement with the rate at which oxygen would be required for complete combustion of the glucose which remained after allowing for lactate production. A similar result was obtained at 36° in observations which have been reported elsewhere and are summarized in column II of Table 2. Thus results at both temperatures agree with the assumptions (a) that glucose taken up by the tissue is all completely oxidized, except for the portion converted to lactate, and (b) that this glucose is the only substrate oxidized under resting conditions. Evidence suggesting that the lactate produced by an excised sympathetic ganglion is formed from glucose taken from the bathing fluid has previously been obtained in experiments at 36° (HOROWICZ and LARRABEE, 1958).

B. Rates of Glucose Metabolism during Activity and Recovery

Increase in rates of glucose uptake and lactate output. Effects of activity were studied in twenty-five experiments at 23° using bathing solutions with various values of pH. Results for the individual experiments, all of which were performed according to the same schedule, are shown in Fig. 1. In each case ½ hr at rest was allowed for purposes of equilibration after the preparation was mounted in the apparatus. The bathing fluid was then replaced with a fresh sample, and a 2 hr measurement was made of glucose uptake and lactate output at rest. This was followed by 2½ hours

of stimulation at a frequency of 6 stimuli/sec. (Since the stimulation was turned off for about 12 sec of each minute while the preparation was being immersed in the fluid, as explained under Methods, the average frequency of stimulation was 4·8/sec). During stimulation, the metabolic measurements were divided into an initial period of ½ hr, to permit detection of any changes which might not be maintained over long periods of activity, followed by a 2 hr period. After the end of the stimulation, metabolic measurements were made during a ½ hr period, which was allowed for recovery from activity, followed by a final 2 hr resting period.

The resting metabolic rates corresponding to the periods of stimulation and recovery were estimated by linear interpolation between the initial and final resting rates. This procedure was justified by a few experiments in which the same sequence of metabolic measurements was made, but no stimulation was applied (Fig. 2, column C). In experiments with stimulation all increments above the interpolated resting level were accordingly ascribed to effects of activity. These increments are indicated in Fig. 1 by the areas with diagonal shading in the initial activity periods, by solid shading in the long activity periods, and by the upper unshaded areas in the recovery periods.

The main conclusion to be drawn from Fig. 1 is that the rates of glucose uptake and lactate output increased during neuronal activity at 23° with every pH investigated. This confirms the finding of similar increases in earlier observations on ganglia at 36° with a pH of 7·3–7·4 (Horowicz and Larrabee, 1958; Larrabee *et al.*, 1957).

Increase in rate at which glucose is available for oxidation. Additional information was gained by averaging the individual results which have just been considered. In doing this the experiments were grouped into two arbitrarily chosen pH ranges. The two experiments at lowest pH (6·30 and 6·52) were omitted from the averages since this range was not adequately investigated, and two others (at pH 6·80 and 6·90) were also left out, because the extraordinarily large rates of glucose uptake during activity so far exceeded the rates in all other experiments in the same pH range that some error was suspected.

In addition to the rates for glucose uptake and lactate output, the differences between these two rates were calculated for each period in each experiment and were also averaged. As previously explained this difference is a measure of the rate at which glucose remains available for possible oxidation or other uses after allowing for lactate production. Provisionally it may be interpreted as a measure of the rate at which glucose is actually oxidized, in view of evidence presented earlier in connexion with the resting metabolism. Results of all calculations are plotted in Fig. 2, using the same conventions as were employed for Fig. 1.

A significant finding revealed in Fig. 2 is an increase during stimulation in the portion of glucose not accounted for as lactate (line 3). This was found in both pH ranges. The present results, obtained at 23°, thus stand in contrast to those previously found at 36°, where there was no increase in glucose available for oxidation during repetitive activity (Horowicz and Larrabee, 1958). In other words some or all of the extra oxygen consumed during activity may be used to burn glucose at 23°, even though this did not occur under the experimental conditions employed at 36°. Quantitative comparisons with oxygen uptake will be made later in this paper.

Effects of changes in pH *on metabolic rates.* Reduction in pH of the bathing fluid caused a decrease in the extra amount of glucose consumed in connection with repetitive activity during the second (2 hr) period of stimulation and also during the recovery period (line 1 of Fig. 2). There was also a decrease in the extra lactate production ascribed to activity in all periods (line 2). Since glucose uptake was affected less than lactate production, there was a resulting increase in the extra glucose which remained after allowing for the extra lactate put out, especially during first ½ hr of stimulation (line 3). According to the assumption that this

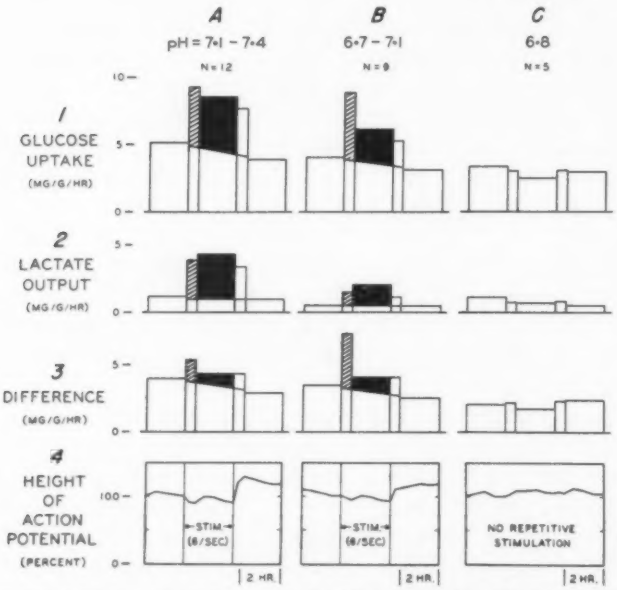

FIG. 2. (1) Average rates of glucose uptake, (2) average rates of lactate output, (3) averages of the differences between these rates, and (4) average heights of the postganglionic action potentials evoked by supramaximal stimulation of the preganglionic nerve. Column A (high pH range) includes all experiments shown in the upper part of Fig. 1. Column B (low pH range) includes all in the lower part of Fig. 1, except experiments at a pH of 6·90, 6·80, 6·52 and 6·30, which were omitted for reasons given in the text. Column C represents five control experiments without repetitive stimulation. All observations at 23°.

portion of the glucose is available for oxidation, the observation implies that oxidation of glucose may have increased more during activity at the lower than at the higher pH. Thus reduction of pH appears to continue a trend, started by reduction of temperature, towards a more complete fulfilment of oxidative substrate requirements by glucose alone.

Time course of the increases in rates. The average rate of glucose uptake was higher during the first ½ hr of repetitive activity than during the subsequent 2 hr of continued stimulation (Fig. 2). This was especially evident at reduced pH, where the average increase in glucose rate above the resting level fell to about half the initial increase in rate. Since lactate production showed no corresponding decline, there was also a falling off in rate for the glucose remainder (Fig. 2, line 3).

Failure to maintain the initial high metabolic rates could not be ascribed to failure of ganglion calls to respond during the long-continued stimulation, since

there was no corresponding decline in height of the postganglionic action potentials (Fig. 2, line 4). Moreover, control experiments without stimulation revealed no tendency for metabolic rates to be higher when calculated from ½ hr periods of measurement than from two-hour periods (Fig. 2, column C). Thus the decline during continued activity does not appear to be an artifact of procedure, but rather a metabolic readjustment developing during neuronal activity continued at constant frequency. Whether this readjustment is characteristic of the tissue, or whether it represents depletion of some material not regenerated under conditions of excision, cannot be determined from the available data. In any case it is relevant that no decline of similar proportion has ever been observed in the rate of oxygen uptake.

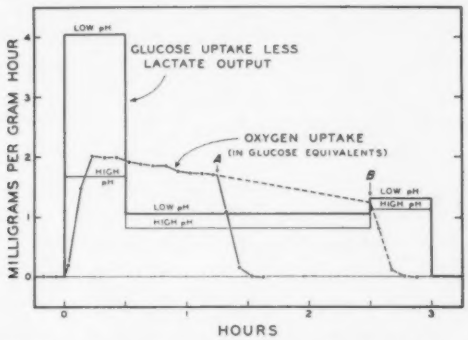

FIG. 3. Time course of metabolic increments above the interpolated resting rates. Repetitive preganglionic stimulation was started at 0 hr in all experiments. When measuring glucose and lactate, stimulation was stopped after 2·5 hr, at B. When measuring oxygen uptake, stimulation was stopped after 1·25 hr, at A. A dotted line has been drawn to indicate the course which oxygen uptake would be expected to follow during 2·5 hr of stimulation, on the assumption that the decline would continue at a constant rate and that recovery after stopping stimulation would follow the same time course as after 1·25 hr of activity. For the glucose and lactate plots, individual experiments were grouped in the same way as for Fig. 2. ('High pH' = 7·4 to 7·1; 'low pH' = 7·1 to 6·7.) The oxygen plot was obtained by averaging the four runs on two preparations shown individually in Fig. 6. Average frequency of stimulation was 4·8/sec for the glucose and lactate measurements, 4·6/sec for the oxygen measurements. 23°.

In Fig. 3, for example, the activity increment in oxygen uptake is compared with the increment in glucose remainder available for oxidation. The rate of oxygen uptake slowly declined during stimulation, but this was not at all comparable to the much greater decline in the rate of glucose metabolism.

When stimulation was stopped, glucose metabolism did not immediately return to the resting level. In fact the average rate of glucose uptake and the rate for the glucose remainder were about the same during the first ½ hr after stopping activity as during the immediately preceding 2 hr of activity. In several of the individual experiments there was actually evidence of an acceleration during recovery above the preceding rates (Fig. 1). Thus transients in glucose metabolism after the end of activity, as well as at the beginning, deserve investigation with methods affording better time resolution.

Finally we may note a contrast in recovery between glucose and oxygen metabolisms. Oxygen uptake returned much more rapidly to the resting level when activity ceased. Moreover, no indication of a terminal acceleration in ganglionic oxygen uptake has ever been observed (Fig. 3 and LARRABEE, 1958).

C. Total Increment in Glucose Metabolism Associated with Activity

A basic question under investigation in these experiments is whether glucose can be the only substrate oxidized in active as well as resting neurones. Since glucose and oxygen metabolisms have been found in good stoichiometric balance in a resting ganglion at all pH and temperature ranges so far investigated, the question may be resolved during activity by comparing the increments above the resting levels. This has been done in Fig. 3, for example. This figure shows that the rate at which extra glucose was made available for oxidation fell below that required for the observed increase in rate of oxygen uptake after the first ½ hr of prolonged activity. This implies oxidation of some endogenous material. On the other hand, glucose was initially made available in great excess. This opens the possibility that the endogenous material oxidized during the later part of the activity might actually be some of the glucose taken up early in activity and retained intracellularly, perhaps not as glucose itself, but as some metabolic product. Moreover, much of the extra glucose consumed during the recovery period immediately following stimulation was not recovered as lactate; this glucose may therefore have served to replace endogenous materials 'borrowed' for oxidation during the later portions of the activity. This possibility was suggested by Dr. ALEXANDER GEIGER (1958), who has seen evidence of such a phenomenon in perfused cat brain. Thus, although the rates of glucose utilization and oxygen uptake are clearly not in balance at all times throughout a period of activity, the possibility remains that any intracellular substrates which have been oxidized may be rebuilt from exogenous glucose during recovery under the conditions illustrated in Fig. 3. The total requirements for oxidative substrate during activity may in this sense be fulfilled by glucose taken up from the cellular environment.

The proposed storage of glucose derivatives and the replacement of 'borrowed' endogenous substrates cannot be directly tested by present methods. But data are available for investigating the possible consequence that the total extra glucose made available for oxidation may be sufficient to account for the total extra oxygen consumed, if temporal pattern is disregarded. In Fig. 2, line 1, the total extra glucose consumed during activity is equal to the sum of the three areas above the line of interpolated resting rate. The total extra lactate formed, and the total remaining glucose not converted to lactate are similarly delineated by appropriate areas on lines 2 and 3. The rest of this paper will be mostly concerned with analysis of these total amounts of extra metabolism associated with 2½ hr of activity, including recovery therefrom.

The total extra glucose metabolism evoked by activity is shown for the individual experiments in Fig. 4A. The length of each vertical line represents the total extra glucose consumed. In order to indicate how this glucose was partitioned between lactate and other metabolic products, the amount of lactate produced is plotted downwards from the line of zero ordinate, and the difference between the glucose consumed and the lactate produced has been plotted upwards. The upper part of the figure thus indicates the amount of glucose remaining available for possible oxidation and other uses.

The total extra glucose consumed during activity declined with pH, as indicated by the total lengths of the lines in Fig. 4A. This trend was statistically significant according to *t*-test of the difference between the averages of the high and low pH

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groups (Table 3). Even more striking was the decline in amount of extra lactate produced as the pH was lowered. This was also statistically significant. This adjustment of lactate production is in the same direction as that observed in resting preparations and might serve a useful purpose in regulation of intracellular pH. On the other hand the change observed in the portion of extra glucose which was not accounted for as lactate could not be considered significant.

Despite considerable variations in the amount of extra glucose consumed in connexion with activity, the products of this additional glucose were often equally divided between lactate and other substances, especially at pH values below 7·1.

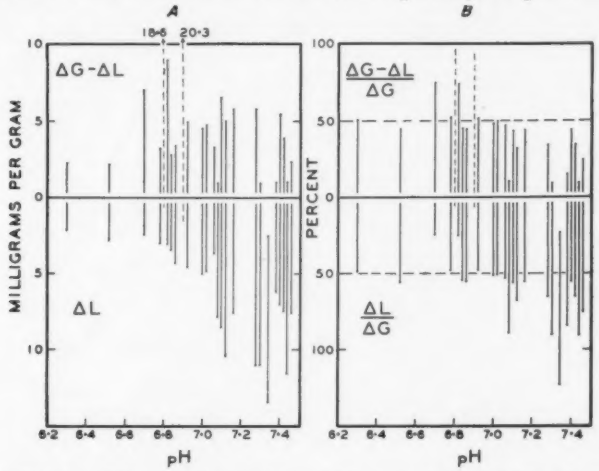

FIG. 4. Partitioning of the extra glucose consumed in connexion with 2·5 hr of activity, shown as a function of pH. Each vertical line represents one experiment. Position of the lower ends of the lines indicate the total extra lactate produced. Position of the upper ends indicate the total extra glucose consumed minus the total extra lactate produced. A—absolute amounts associated with the activity. B—the same amounts expressed as fractions of the total extra glucose consumed. All experiments shown in Fig. 1 are included, but two questionable ones are represented by dotted lines. 23°.

This is best seen in Fig. 4B. At higher pH values there was a tendency for an increased percentage of the extra glucose to go to lactate.

The precision with which the 50-50 division frequently occurred at pH values below 7·1 might suggest the operation of some biochemical mechanism for channelling successive molecules of pyruvate alternately into reduction and into oxidation. However, this symmetrical partitioning was not found in the resting uptake: at rest only a small fraction of the consumed glucose appeared as lactate (Table 1). Thus a simple explanation for the partitioning of the extra glucose of activity presumably could be derived only by supposing that the 'resting' and 'activity' portions of the glucose uptake were metabolized through different routes. Another difficulty is that the symmetrical partitioning of glucose was found only in the total extra metabolism associated with activity. No such simple rule applied to the extra glucose consumed during the individual periods of measurement.

D. Rate of Oxygen Consumption During Activity

Because discussion of the foregoing results will concern comparison with the rate of oxygen uptake as well as with previous experiments conducted at higher

TABLE 3.—EXTRA METABOLISM ASSOCIATED WITH 2·5 HR OF REPETITIVE ACTIVITY AT 23°

	I	II	III	IV
pH	7·45–7·10	7·09–6·45	Difference	*t*-test of difference
		mg/g dry weight		
A. Extra glucose taken up	12·20 ± 0·80 (12)	8·62 ± 0·66 (9)	3·58	$P < 0·01$ (significant)
B. Extra lactate put out	9·13 ± 0·66 (12)	3·80 ± 0·30 (9)	5·33	$P < 0·01$ (significant)
C. Difference between extra glucose taken up and extra lactate put out	3·07 ± 0·80 (12)	4·82 ± 0·69 (9)	1·75	$P > 0·1$ (not significant)
		μmoles/g dry weight		
D. Oxygen equivalent of difference C	102 ± 27 (12)	161 ± 23 (9)		
E. Extra oxygen taken up	142 ± 15 (4)			
F. Oxygen excess (=E − D)	40	−19		

Mean ± S.E.M. Numbers of preparations in parentheses. Average frequency of stimulation = 4·8/sec for glucose and lactate measurements (see text), 4·6/sec for oxygen measurements.

Columns III and IV refer to the difference between the average amounts in the two pH groups.

The extra oxygen taken up (item) E was estimated from the results of four experiments by multiplying the observed peak increases in rate of uptake by 0·85 to allow for a decline during prolonged activity, and then by 2·5 to obtain the total increase for 2·5 hr of activity. The factor of 0·85 was used because the increase was found to decline linearly with time in the average results of four runs in two experiments in which the preganglionic nerve was stimulated at 4·6/sec for 1·25 hr, reaching a value at the end of this stimulation which was 85 per cent of the peak value (see Fig. 3).

temperature, it will next be necessary to consider the extent to which oxygen consumption is altered by changes in temperature and pH.

Slowing of the resting oxygen uptake by reduction of temperature is illustrated for two preparations by the dashed lines in Fig. 5A. By contrast with this resting rate, the increment in oxygen uptake accompanying activity did not decline continuously with temperature, but instead had a maximum value in the vicinity of 30° (solid lines in Fig. 5A). The height of the postganglionic action potential also tended to be maximal at about the same temperature (Fig. 5B). Therefore the peak of the metabolic response may in part reflect a maximum in the number of ganglion cells

responding to each preganglionic volley, but the changes in response do not appear to be sufficiently large to account for all changes in the oxygen increment in the range from 25° to 35°. Possibly the alterations in electrical responses were a result rather than a cause of the oxidative changes. It is also relevant that the increment in oxygen uptake accompanying activity is relatively independent of temperature in peripheral nerves of rats (CRANEFIELD, BRINK and BRONK, 1957).

Considering next the possible effects of pH, it has been stated earlier in this paper that the resting rate of oxygen uptake did not seem to vary characteristically with pH at 23–25°, although this independence has not been demonstrated with

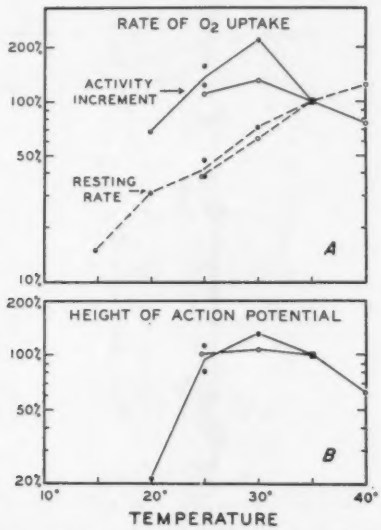

FIG. 5. Rate of oxygen uptake (A) and height of the postganglionic action potential (B) as functions of temperature. Two ganglion preparations are represented by different symbols. All data are expressed as percentage of values at 35°. In one experiment the increase in rate of oxygen uptake was measured at the end of 20 min of stimulation at a frequency of 4·6/sec at each temperature, in the other at the end of 10 min at 4/sec. In each case the height of the postganglionic action potential was measured 10 min after starting the repetitive stimulation. Bathing solution was equilibrated with 5% CO_2, 95% O_2 at each temperature; no compensation was made for change of pH with temperature.

great precision. The activity increment in oxygen uptake was more carefully investigated and was found to be independent of pH in two well-controlled experiments (Fig. 6). In one experiment a ganglion was first stimulated at pH 7·4 and then at pH 6·9. In the other the order of pH applications was reversed. In both experiments the increment in oxygen uptake was somewhat smaller during the second stimulation than during the first. The small differences in oxygen uptake could thus be ascribed to a decline with time, rather than to the changes in pH.

DISCUSSION

Substrates for oxidation. Resting oxygen uptake could be accounted for in excised ganglion preparations under all conditions investigated by assuming that glucose taken from the bathing fluid was the only substrate oxidized (e.g. Table 1). By contrast, previous investigations at 36° have shown that some material endogenous to the excised ganglion was burned by the extra oxygen used during activity, since

all the extra glucose taken up by the tissue was accounted for as lactate (LARRABEE and HOROWICZ, 1956; HOROWICZ and LARRABEE, 1958). Analysis of the present experiments, however, reveals that at 23° much or all of the activity increment in oxygen can be accounted for by assuming oxidation of glucose (Table 3, columns I and II).

In preparing Table 3 only the total extra metabolism associated with a given amount of activity has been considered, rather than the rates of this metabolism, in order to avoid problems which have been already discussed in relation to the temporal pattern of the metabolic increases. It is evident from the table that in the

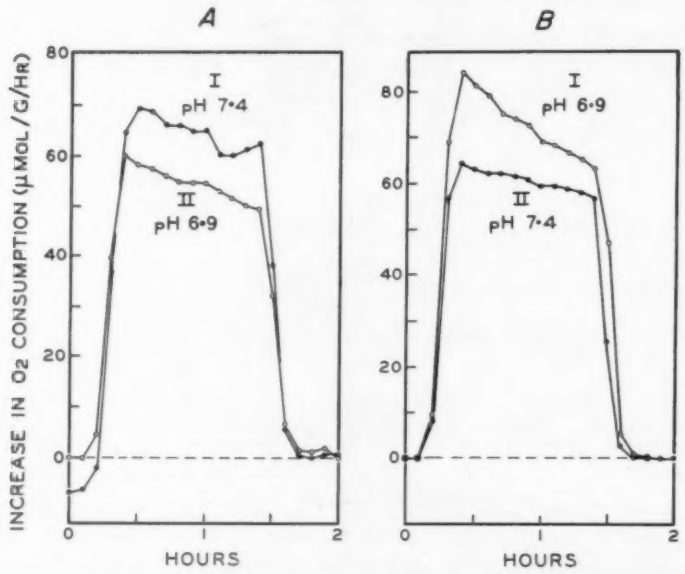

FIG. 6. Increase in rate of oxygen consumption by sympathetic ganglia, immersed in bathing fluids with various values of pH, during activity caused by supramaximal stimulation of the preganglionic nerve at a frequency of 4·6/sec. A and B refer to different preparations. I and II indicate sequence of observations in each. 23°.

higher pH range at 23° the extra glucose available for oxidation accounted for a major fraction of the extra oxygen associated with activity. Moreover, in the lower pH range the extra glucose available actually averaged somewhat in excess of the extra oxygen.

Metabolic increments are summarized graphically and brought into perspective with the resting metabolism in Fig. 7. In this case all data including that at 36° are presented on a 2·5 hour basis. Resting glucose metabolism is represented by filled circles. Increments observed in this metabolism when stimulating are shown by solid arrows. Both are resolved into the usual two fractions of lactate produced (ordinate) and glucose remainder (abscissa). Oxygen metabolism is also represented by dashed arrows drawn parallel to the scale of abscissa, after conversion into the quantity of glucose which could be oxidized by the amount of oxygen consumed. The oxygen arrows start at abscissae corresponding to resting oxygen uptake and have lengths equal to the increment caused by activity. In accordance with results reported above, which indicate that oxygen uptake is independent of pH, a single

oxygen arrow suffices for comparison with the two groups of glucose data at the lower temperature.

A striking feature of Fig. 7 is the relatively small effect of temperature change on the activity increments in glucose uptake and lactate output, compared to the large effect on resting metabolism. A similar constancy in the oxygen increment is also shown and has already been mentioned above. The major effect of temperature reduction on the activity increment in metabolism is a shift in partitioning of the extra glucose consumed, so that a greater fraction is available for uses other than lactate production. This altered partitioning is demonstrated by the relative

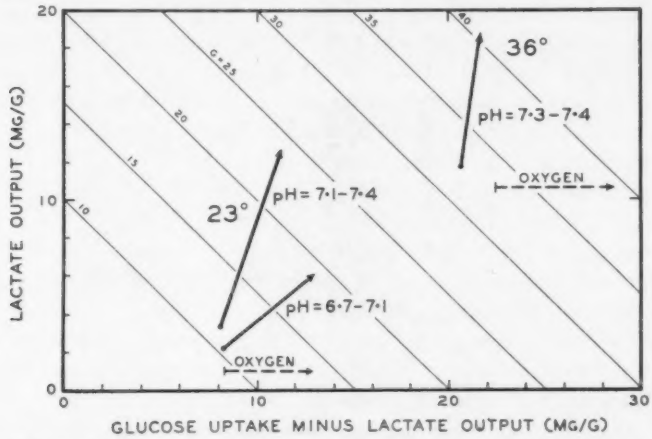

FIG. 7. Diagramatic representation of ganglion metabolism. Filled circles indicate the amount of lactate produced (ordinate) and glucose consumed less lactate produced (abscissa) during 2·5 hr at rest. Solid arrows show the total extra amounts associated with 2·5 hr of activity. Positions of filled points and lengths of solid arrows with respect to diagonal lines indicate glucose uptake; numbers on these lines indicate total glucose taken up (mg/g). Also shown in accordance with the scale of abscissae is the glucose equivalent of the amount of oxygen consumed during 2·5 hr at rest (short vertical bars) and the glucose equivalent of the extra oxygen associated with 2·5 hr of activity (dashed arrows). Frequency of preganglionic nerve stimulation averaged 4·8/sec for all measurements, except for a frequency of 4·6/sec in the oxygen measurements at 23°. Data at 36° were obtained from LARRABEE (1958) and HOROWICZ and LARRABEE (1958).

slopes of the various solid arrows in Fig. 7, which turn more towards the glucose remainder as temperature is lowered, and still more as pH is also reduced at the lower temperature. In the last case especially, enough glucose appears to be available for oxidation to support the increased oxygen uptake without resort to other substrates.

The change, caused by alteration of pH, in the partitioning of the extra glucose associated with activity was statistically significant at the 1% level. This was shown by calculating the ratio of extra lactate to extra glucose in each experiment and applying a *t*-test to the difference between the averages of the two pH groups.

Speculation on the mechanism of substrate change. Starting with an excised tissue which is entirely dependent on an endogenous substrate for the extra oxidation that accompanies activity when physiological conditions are simulated, it has been converted, by lowering temperature and environmental pH, to a tissue which appears to meet all its oxidative needs by the use of exogenous glucose. In the first circumstance the experimental results on ganglia resemble those of other investigators

on nerves excised from frogs and rabbits and on excised or perfused animal brain. In the second case the findings are more reminiscent of those on normally circulated human brain *in situ*.

In considering possible mechanisms of these metabolic differences, it is convenient to assume the existence of three more or less independent sets of regulatory factors. The first is assumed to determine the amount by which oxygen uptake increases during activity, the second the amount by which glucose uptake increases, and the third the partitioning of the extra glucose between various possible metabolic destinies. The alterations in this partitioning are most relevant in present context. If we assume (a) that glucose is metabolized according to the Embden–Meyerhof scheme and (b) that the glucose not accounted for as lactate is mostly oxidized, then the partitioning can be considered as a result of the relative values of the velocity constants controlling pyruvate reduction on the one hand and pyruvate oxidation on the other. Thus the shift towards oxidation at low temperature and pH could result either from impairment of pyruvate reduction (e.g. inhibition of lactate dehydrogenase), or from improvement of conditions for oxidation (enhancement of the pyruvate oxidase system). Either of these fundamental changes could increase the amount of extra glucose oxidized during activity and consequently could reduce the amount of other substrates that must be used. Whether one of these mechanisms indeed causes the observed alterations is at present unknown.

Speculations on metabolism in situ. In speculating on ganglionic metabolism under natural conditions in the body, it would be most obvious to assume that natural conditions are best simulated in the excised tissue by working at physiological temperature and pH. The appropriate pH for such investigations is not easily chosen, however, in view of evidence that in tissue such as brain the extracellular pH differs from that in blood (ELLIOTT and JASPER, 1949). Moreover, a gradient of pH may be established in excised tissue by the outward diffusion of lactic acid and CO_2, so that the immediately extracellular pH does not necessarily equal that of the bathing fluid. Furthermore, if it nevertheless be assumed that physiological conditions are best simulated by a temperature of 36–37° and a bathing fluid with pH of 7·3–7·4, the significance of the acceleration of glucose uptake during activity is unexplained, since the extra glucose does not seem to be used for oxidative generation of an appreciable amount of energy under these circumstances.

One way of resolving these questions is to assume that blood contains a natural inhibitor of pyruvate reduction, or an accelerator of pyruvate oxidation, which is lacking from the bathing fluid employed for excised tissues. Then extra pyruvate formed during activity *in situ* might be partly oxidized, as in an excised preparation immersed in bathing fluid at reduced temperature and pH. The extra glucose could then serve for oxidative energy production. We know of no natural inhibitors of pyruvate reduction like those proposed. On the other hand, pyruvate oxidation requires several vitamins and other materials not included in the bathing fluid. A relevant and, at present, unanswerable question is whether lack of any of these substances could become evident within the few hours required for our experiments.

These speculations thus imply that normally the rates of glucose uptake and oxygen uptake may be harmoniously adjusted during activity, perhaps by intracellular concentrations of phosphate and phosphate acceptors, so that appropriate amounts of carbohydrate are provided for oxidation. These regulating factors may then

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continue to make similar adjustments in rates of glucose and oxygen uptake even when there is derangement of the reactions by which glucose or its products are fed into the oxidizing system, as under conditions of excision. If this be true the experimental derangement can apparently be corrected by lowering temperature and pH.

But these are merely hypotheses and only serve the purpose of suggesting further investigations. Perhaps our most significant finding is that glucose metabolism can be modified in a controlled manner in a particular preparation, so as to resemble either of two contrasting metabolic relationships found by other investigators in various nervous tissues. This encourages a hope of eventually reconciling divergent points of view and of explaining the basis for seemingly conflicting results.

SUMMARY

The rates of glucose uptake, oxygen uptake, and lactate output were measured at 23° in sympathetic ganglia excised from rats and bathed in fluids with pH varying from 6·3 to 7·4. Measurements were made with the ganglion cells at rest and also during activity induced by supramaximal electrical stimulation of the preganglionic nerve at an average frequency of 4·8/sec. Heights of action potentials in the postganglionic nerve were measured repeatedly during each experiment. Results were compared with those previously obtained in experiments at 36°.

Reduction of temperature from 36° to 23° caused the expected decreases in the resting rates of glucose uptake, oxygen uptake, and lactate output. At rest at both temperatures the observed rate of oxygen uptake was in good agreement with the rate required for complete combustion of the glucose consumed after allowing for lactate production.

Neuronal activity was accompanied by increases in all three of these metabolic rates at 23°, as had been previously found at 36°. The magnitude of the increases were affected much less by the change in temperature than were the resting rates. The extra amount of oxygen consumed during activity exhibited a maximum at about 30°.

At 23° lowering the pH (from 7·4–7·1 to 7·1–6·4) was accompanied by a significant reduction in the resting rate of lactate production. There were also significant reductions in the extra amounts of glucose consumed and lactate produced in connexion with repetitive activity. No significant effects of pH were found in the rate of oxygen uptake, either in resting preparations or during activity.

When the metabolic rates were increased by activity at 23°, the rate of glucose uptake was accelerated more than the rate of lactate output, so that some of the extra glucose remained available for possible oxidation. The percentage available was greater at low pH than at high. These results contrast with those of earlier experiments at 36°, in which the rate of lactate production was increased by activity as much as was the rate of glucose uptake.

The rate of oxygen uptake rose rapidly to a new level at the start of stimulation, declined but slightly when the activity was long continued, and promptly returned to the resting level when activity was stopped. The rates of glucose uptake and lactate output also increased at the start of activity, but the glucose rate declined considerably during continued stimulation. After activity ceased glucose and lactate rates returned much more slowly to the resting level than did oxygen uptake; sometimes there was actually a terminal transitory increase in the glucose rate. Thus the rate of oxygen

uptake was not in balance with glucose metabolism at each instant throughout a period of activity and recovery thereform. However, when temporal distribution was disregarded, the total extra oxygen consumed could be largely accounted for by the total extra glucose taken up, after allowing for the total extra lactate produced. In this sense a balance was found between the total glucose metabolsim and the total oxygen uptake associated with activity, the best agreement occurring at reduced pH.

Thus by varying temperature and pH the metabolism of an excised sympathetic ganglion could be modified in such a way that under one circumstance (36°, pH 7·3–7·4) oxidations which accompanied activity utilized some unidentified substrate which was endogenous to the excised tissue, while under other conditions (23°, pH 6·4–7·1) exogenous glucose accounted for all oxygen consumed during activity as well as at rest.

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THE DEPOSITION AND DISPOSAL OF (4-^{14}C) CHOLESTEROL IN THE BRAIN OF GROWING CHICKENS

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MUCH has been learned in recent years through the use of electron microscopy and X-ray diffraction studies about the laminated structure of the myelin sheath (FERNÁNDEZ-MORÁN and FINEAN, 1957). Such studies have disclosed its distinctively organized character, and the likelihood that the various types of protein and lipid molecules recoverable from it by chemical extraction are mutually linked in a highly orientated manner. Little biochemical work has yet been done, however, to determine whether this apparent stability of internal structure in the sheath has as its counterpart any tardiness in the metabolic 'turnover rate' of any or all of its known constituent materials.

Of the various lipids typical of myelin (JOHNSON, MCNABB and ROSSITER, 1948), cholesterol possesses several notable advantages for such 'turnover' studies. In addition to being quantitatively one of the principal members of this group of lipids, it is well characterized chemically, readily extractable and capable of accurate estimation. It can furthermore be obtained labelled with radioactive carbon, so that the presence and duration of persistence of this identifiable form of the compound can be ascertained with considerable accuracy.

In the following study, radioactive cholesterol in trace amounts of high specific activity has been introduced into newly-hatched chickens at a time when myelination is proceeding with great rapidity. By extracting their brains for lipids at various intervals afterwards, it has been possible to determine the extent to which this cholesterol had become incorporated in the central nervous system and to gain some indication of its 'turnover rate' from the rate at which it subsequently disappears.

MATERIALS AND METHODS

Animals. The chickens used were a cross-bred strain of Light Sussex and Rhode Island Red fowls. After hatching, they were kept at incubator temperature for 2 weeks. Both series of chickens used were given free access to water after hatching, but whereas with the first, food was given within the first day or two, with the second, feeding was delayed until the fifth day to encourage the more rapid absorption of the residual yolk sac contents. In the newly-hatched chicken, the yolk sac can be palpated, and the injection of the cholesterol-stearate emulsion (0·25 ml) was made through the abdominal wall directly into its interior. This dose contained 60 μg radioactive cholesterol (3 μc).

Cholesterol-stearate emulsion. The (4-^{14}C) cholesterol (50 μc/mg) was obtained from the Radiochemical Centre, Amersham. It was emulsified in a solution of sodium stearate (0·1%) by the method described by BERGSTRÖM and WINTERSTEINER (1941) to form a preparation having an activity of 12 μc/ml.

Preparation of organ extracts. Chickens were killed by decapitation and their blood collected in heparinized tubes. The organs required were removed, blotted dry and weighed. Samples of about 100 mg were dried at 110° for 2 hr for dry-weight determinations. The remaining tissue was homogenized with ethanol–ether in a glass Potter–Elvehjem homogenizer. The homogenate was extracted

on a water bath at 60° for at least 10 min, filtered through a fat-free paper, and adjusted to volume in a standard flask.

The blood was centrifuged and a suitable measured volume of plasma (usually 5 ml) added slowly to 15 volumes of ethanol–acetone. The mixture was warmed to 60° for 10 min and then filtered. The precipitate was washed with alcohol–acetone and the washings combined.

Determination of cholesterol

(a) *Reagents.* Acetone–ethanol 1 : 1 v/v. Ethanol–ether (anaesthetic) 3 : 2 v/v. Digitonin solution: 0·5% (w/v) in 50% ethanol. Alcoholic KOH: 10 g potassium hydroxide dissolved in 40 ml ethanol. Colour reagent: 1 part concentrated H_2SO_4 added to 20 parts ice-cold acetic anhydride (A.R.)

(b) *Procedure.* Total cholesterol was determined on extracts hydrolysed with alcoholic KOH Alcoholic KOH (0·1 ml) was added to suitable volumes estimated to contain about 500 μg cholesterol and the mixture kept at 50° for 1 hr. The alkaline extract was then neutralized with 10% acetic acid using phenolphthalein as indicator. Digitonin solution (1 ml) was added to the neutralized extract. For the determination of free cholesterol, 1 ml digitonin solution was added to the original untreated extract. The cholesterol digitonide was allowed to precipitate either by standing for at least 5 hr or by the addition of aluminium hydroxide gel (OBERMER and MILTON, 1937) and centrifugation. The precipitate was washed with ether and re-sedimented. The digitonide was dissolved in 1 ml glacial acetic acid. A volume of 4 ml of the colour reagent was then added, and after shaking the mixture was left for 25 min in the dark at 25°. The optical density was determined in a Spekker absorptiometer using a red filter (Wratten 8). Standard cholesterol solutions were estimated concurrently with the unknown samples.

(c) *Control estimations.* Six estimations of the same preparation of cholesterol stearate containing 500 μg of cholesterol gave a standard error of ± 5 μg. Six specimens of liver from six embryos on the eighteenth day of incubation gave the following results when expressed as the total amount in the whole liver:

Free cholesterol	$0{\cdot}98 \pm 0{\cdot}016$ mg
Ester cholesterol	$2{\cdot}90 \pm 0{\cdot}47$ mg

Assay of radioactivity. For the estimation of radioactivity, the cholesterol was precipitated as the digitonide. Radioactivity was determined by the technique described by POPJÁK (1950). Some of the cholesterol (6 mg) was precipitated as the digitonide and collected by filtration on a paper disc 1 cm in diameter lying in a perforated polythene planchet in a small dismountable Büchner funnel. The collected digitonide, together with the paper disc and planchet, were dried overnight over paraffin chips. The counts were made with a mica end-window EHM2S tube. Duplicate specimens were usually made and both were counted over a 60 min period. Cholesterol (6 mg) to which a small quantity of radioactive cholesterol had been added and prepared for counting as above gave a corrected count of 2163 counts/min per 0·1 μc. Estimates of total organ radioactivity of the brain and liver showed that at their maximum the former incorporated 0·2 per cent and the latter 23 per cent of the dose given.

RESULTS

The cholesterol contents of the liver and brain

The amounts of cholesterol present in the liver and brain of chickens of various ages before and after hatching are set out in Table 1. During the terminal stages of incubation and the first two days after hatching, there is a rapid absorption of cholesterol and other lipids from the quickly shrinking yolk sac. Much of this cholesterol becomes temporarily deposited in the liver, which for a few days assumes a brilliant ochre colour and presents histologically the features of intense lipoid infiltration of its parenchymal cells. From the metabolic standpoint, a feature of interest in this brief phase of storage is the rapid alteration in the proportions of the cholesterol present in its esterified and free forms; about the time of hatching, roughly nine-tenths is esterified, but a fortnight later, the proportion is almost reversed.

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TABLE 1.—CHOLESTEROL CONTENT OF BRAIN, LIVER AND PLASMA IN CHICKENS AT VARIOUS STAGES OF DEVELOPMENT

Age of chick (days)	Brain			Liver				Plasma	
	Wet weight (g)	Dry weight (%)	Cholesterol free (mg)	Wet weight (g)	Dry weight (%)	Cholesterol ester (mg)	Cholesterol free (mg)	Cholesterol ester (mg)	Cholesterol free (mg)
−6	0·470	12·1	1·9	0·233	17·7	1·2	0·6		
−3				0·349		2·9	1·0		
−2				0·538	28·0	18·1	2·6		
−1				0·541		33·2	3·4	5·3	0·8
0	0·955	16·9	6·7	0·660	36·6	39·9	3·3	3·3	1·3
+1				0·938		55·3	5·9	1·5	1·1
+2	0·927	17·1	7·7	1·121	34·0	68·0	5·1	2·1	0·5
+4				1·530		25·1	14·0		
+5	0·923		10·5	1·689		29·0	10·3		
+7	1·000	18·4	10·3	2·471	30·0	26·0	9·9	1·8	0·6
+10	1·344	20·1	17·3	2·400	28·2	18·2	5·7		
+15	1·414		13·4	4·533		1·0	9·5	1·0	0·4
+18								1·0	0·5
+22	1·452	21·1	16·8	5·051	28·3	3·9	12·3		
+29	1·685	20·5	19·6	7·145	23·3	0	18·9		
+45	2·873	20·3	38·3	17·052	35·3	0	50·0	0·4	0·3
+120	2·803		42·5	32·200		0	111·0	0·5	0·4

These results are in close agreement with those of ENTENMAN, LORENZ and CHAIKOFF (1940).

In the brain, the cholesterol, which by the end of incubation is almost wholly in the free form, increases about six-fold during the first 4 months of post-embryonic life. At hatching, it forms about 4 per cent of the dry weight of the brain, but by the time that this organ has reached adult size, this percentage has almost doubled. The value found in a bird of 4 months (7·5 per cent of the dried weight) is almost the same as that recorded for the cerebrum of adult fowls by KAWAKITA (1956).

The radioactivity of cholesterol extracted from the liver and brain

The radioactivities of the digitonide precipitates obtained from the brain, liver and plasma at various intervals after hatching are shown in Fig. 1. Since some of this radioactivity might be supposed to result from an incomplete exsanguination of the organs at the time of removal, an estimate was made by the employment of intravenously injected ^{131}I-labelled homologous plasma proteins of the amount of residual blood that they might contain at the time they were homogenized. The results for two birds examined in this way, one 4 and the other 12 weeks old, are given in Table 2. From these figures, it can be seen that at both ages, the excised liver contained approximately one-fifth of its weight of blood, though because of the exceptional permeability of the capilliaries in this organ, this estimate may be too high. At neither age, however, did the quantity in the brain rise above one-sixtieth. Thus, although some of the radioactivity of the liver must be assigned to retained blood, hardly any of that of the brain can be attributed to this contamination.

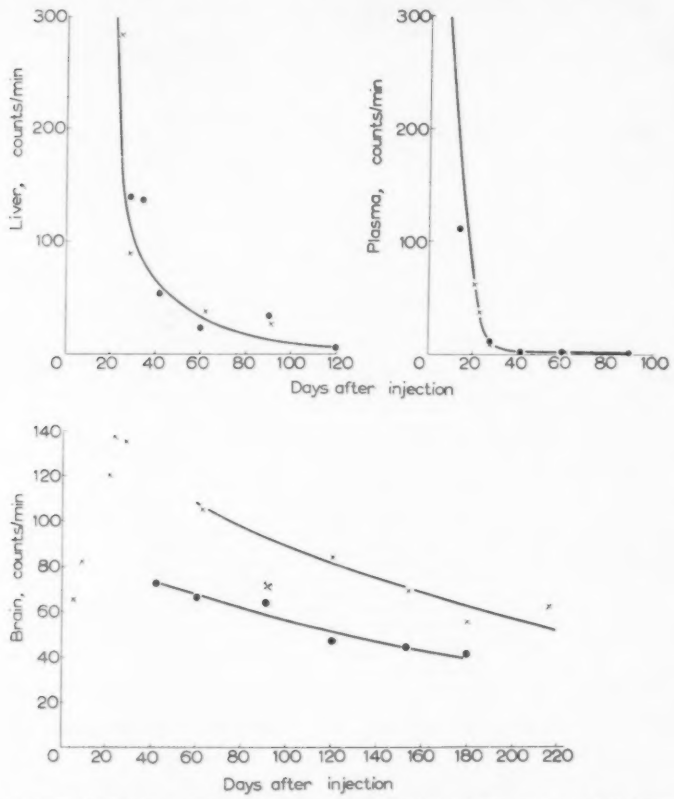

FIG. 1. Levels of radioactivity (expressed as counts/min per whole organ or per ml of plasma) in the brain, liver and plasma of developing chickens at various intervals after the administration of radioactive cholesterol. Two groups of chickens were given 3 μc of (4-^{14}C) cholesterol, the first group (●) were fed immediately after hatching and the second group (×) were starved for five days.

TABLE 2.—TOTAL BLOOD VOLUME IN CHICKENS, AGED 28 AND 82 DAYS, AND THAT IN THEIR EXCISED BRAIN AND LIVER AS DETERMINED BY THE INJECTION OF ^{131}I-LABELLED SERUM PROTEIN

Chicken age (days)	28	82
Weight (g)	150	1425
Total blood volume (ml)	17	142
Brain blood volume (ml)	0·022 (0·0138 ml/g)	0·053 (0·0156 ml/g)
Liver blood volume (ml)	1·1 (0·22 ml/g)	7·0 (0·19 ml/g)

Moreover, after the first few weeks, the radioactivity of the plasma has fallen so low that it could make little contribution to that of the organ parenchyma itself.

From Fig. 1, it can be seen that although the radioactive cholesterol is incorporated rapidly in the liver in large amounts during the first few weeks after hatching, it falls very rapidly from its maximum until by the end of 8 weeks only a small fraction of the original quantity remains. On the other hand, the rate of uptake and of disappearance of the radioactive cholesterol in the brains of both series of chickens is very much more gradual.

DISCUSSION

Two pieces of evidence, both of an indirect nature, have led to the belief that the cholesterol of the myelin lipids has a relatively slow rate of 'turnover'. The first was obtained by BLOCH, BERG and RITTENBERG (1943) who fed cholesterol labelled with deuterium to an adult dog for 3 days; although at the end of 6 days the marked material could be recovered from the other organs examined, none was found in the brain or spinal cord. The second was obtained by WAELSCH, SPERRY and STOYANOFF (1940) who first injected and then fed adult rats with heavy water for periods of from 4 to 7 days. No significant amounts of deuterium were found in the unsaponifiable lipids of the brain, although appreciable quantities of this isotope had by that time become incorporated in the unsaponifiable lipids of the liver and intestines. When they administered heavy water to newly-born rats, however, the deuterium was promptly incorporated in the unsaponifiable lipids of the brain and in a measure that was inversely related to their age at the time and directly to their current rate of myelin deposition.

The present study differs from these earlier investigations in two material respects. First, radioactive carbon labelled cholesterol has been used and has been administered at a time when the process of myelination is taking place very actively and the amount of cholesterol in the brain is increasing rapidly. The recovery of labelled cholesterol as the precipitated digitonide suggests that, contrary to the statements of certain earlier authors (WAELSCH *et al.*, 1941; FOLCH-PI, 1955), cholesterol may be removed from the circulating blood at this period of development of the chicken and incorporated in the substance of the central nervous system. In the second place, the observations have been continued over a sufficiently long period (6 months) for some estimate to be made of the rate of 'turnover' of this lipid. Its comparative slowness can be seen from the finding that a large proportion of the radioactive cholesterol originally administered is still recoverable from the brain 6 months later, at the time that the bird is almost full grown. It is of interest in this connexion that MCMILLAN, DOUGLAS and MORTENSEN (1957) found that the radioactive cholesterol synthesized in the brains of young rats from ^{14}C labelled acetate, persisted there with little change for many weeks.

Our estimates of the persistence of radioactive cholesterol in the brains of chickens are confined to overall evaluations of its deposition and disposal, and since this substance is an important constituent of cells as well as of myelin, it is not possible to separate any "turnover" rates in these two structures. It may prove that the metabolic processes take place at very different rates, and that were it possible to dissociate the myelin from the cells, the cholesterol might be found to persist much longer in the former than in the latter.

SUMMARY

(1) Radioactive carbon-labelled cholesterol was injected into the yolk sac of newly-hatched chickens and its turnover subsequently studied in the plasma, liver and brain.

(2) The rate of disappearance of radioactive cholesterol was much slower from the brain than from the liver, and from observations extending over the first 6 months after hatching, it is evident that a large proportion of it is still present in the central nervous system after 150 days.

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RELATION BETWEEN SURFACE, RIBONUCLEIC ACID CONTENT AND NUCLEAR VOLUME IN ENCAPSULATED SPINAL GANGLION CELLS*

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WHILE the ribonucleic acid (RNA) in nerve cell bodies under various experimental conditions has been much studied, there is relatively little information available on how the RNA content of nerve cell bodies is related to their dimensions. Information on this point would consequently be desirable.

The object of the present investigation has been to determine the RNA content in individual nerve cell bodies belonging to a population where the cell body size shows great variation, and then from the data plot in what way RNA is related to cell body size. For this purpose freshly dissected spinal ganglion cell bodies in their capsules of satellite cells were used.

It has been previously claimed that, in nerve cells including spinal ganglion cells, the volume of the nerve cell nucleus and the area of the cell body surface are proportional to one another (BOK, 1936). These conclusions were based on a study of fixed material. As it is difficult to exclude shrinkage artifacts when using fixed material (e.g. preferential shrinkage of the cytoplasm of bigger cells and relatively constant shrinkage of the nuclei of all cells) we thought it worth while also to use our fresh, untreated cell bodies for an investigation on this question.

MATERIALS AND METHODS

Encapsulated cells were obtained from four adult rabbits weighing between 3 and 4·5 kg and three young rabbits of 2–2·5 kg. Another five young rabbits were used for the determination of cell dimensions of free cells. The animals were bred and kept in cages and were healthy. They were killed by intravenous air injections. Spinal ganglia were quickly removed and put into cold 0·25 M-sucrose solution.

Single cell bodies, either free or enclosed in their capsules of supporting cells, (hereafter called systems), were dissected out of spinal ganglia in the sucrose solution. The dissections were carried out freehand, under a binocular dissecting microscope (magnification ×36–50) with fine iridectomy knives. The dissection procedure has been described in detail previously (HYDÉN, LÖVTRUP and PIGON, 1958).

The excised cells or systems lying on agar-coated slides in a drop of sucrose solution were measured under a light microscope at a magnification of 560 diameters using a dry lens. Care was taken to have the drops so flattened that they did not exert any lens effect. The two main horizontal diameters of the cell body were measured with the aid of a measuring eye-piece, the vertical diameter by means of focusing the upper and lower visible structures and reading off the micrometer screw. The latter had been calibrated by measuring the movement exchange with the aid of another microscope kept horizontally. The values for vertical movement read off on the micrometer screw were multiplied by the refractive index of the sucrose solution. The mean value of two determinations of the vertical diameter was used. The volume was computed according to the formula: $V = \pi ABC/6$,

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where V is the volume and A, B and C the three diameters. The value for the surface area (S) of the measured body was derived from the volume according to the formula: $S = 4{\cdot}836\ \sqrt[3]{V^2}$, which is an approximation giving errors which may be neglected.

Nuclear volume values were obtained by measuring the two diameters lying in the plane of the section and assuming the third one to be intermediate between these two (i.e. $v = \pi ab\sqrt{(ab)}/6$, where v is the volume of the nucleus and a and b its two measured diameters respectively).

Only systems were used for RNA determinations since it was found that RNA was lost from isolated free nerve cell bodies during isolation and measurement. After the diameter determinations the systems in a minimum volume of sucrose solution were transferred to a cover slip of the dimensions 12 × 32 × 0·17 mm by means of an agar-coated braking pipette. They then adhered to the glass surface as the accompanying liquid evaporated. On the back of the cover slip markings had been made with a mixture of indian ink and albumen to make later localization of the systems possible. Cover slips, each containing six to ten measured systems, were treated with ice-cold N-perchloric acid for 5 min. This treatment was followed by successive 5 min treatments with absolute ethanol and chloroform. The cover slips were then transferred to, and kept in, liquid paraffin at refrigerator temperature (+4°C). RNA determinations were later performed by the method of EDSTRÖM (1958).

RESULTS

The volumetric distribution of free cell bodies was between 25,000 μ^3 and 500,000 μ^3 with a median value of 180,000 μ^3; that of cell bodies within their capsules (systems) between 35,000 and 700,000 μ^3, median value 250,000 μ^3.

The RNA values were distributed between 200 and 2500 $\mu\mu$g with a median value of 1070 $\mu\mu$g. Judging from tissue sections, stained or viewed in ultraviolet light, RNA is predominantly localized in the nerve cell body itself and the contribution of the capsule to these values is probably negligible.

It was soon evident that there was a relation between the size of the system and its RNA content, the larger systems containing more of this substance. Some systems, extracted and afterwards stained, are shown in Fig. 1 together with their corresponding RNA extracts to illustrate this correlation.

As it was found that there is no linear regression of RNA content on volume in systems, these two units have not been plotted against each other here. Instead RNA values have been plotted against surface areas in Fig. 2. As the results from the individual animals did not differ to any significant extent from each other, the values from all the rabbits have been combined. The regression line has been constructed graphically.

To find the regression line according to the method of least squares it would have been necessary to transform the values logarithmically. As this would give a non-linear regression in the present instance the relatively laborious task of fitting a regression line has not been undertaken particularly as it cannot be expected to give any further information of importance.

The equation of the graphically constructed regression is:

$$y = 0{\cdot}68(x - 2620)$$

where y is the RNA content of the system in $\mu\mu$g and x its surface area in μ^2.

Thus, it can be seen that the amount of RNA in the system is proportional to its surface area (minus the relatively small fraction 2620 μ^2).

It would be of interest to know whether a linear regression also exists for RNA on the surface area of the cell body. Although it is possible to determine how much the capsule contributes to the volume of the system, it is difficult from this information to estimate the size of the area of the cell body surface, which has been shown to be irregular submicroscopically (HESS, 1955). Investigations were performed which

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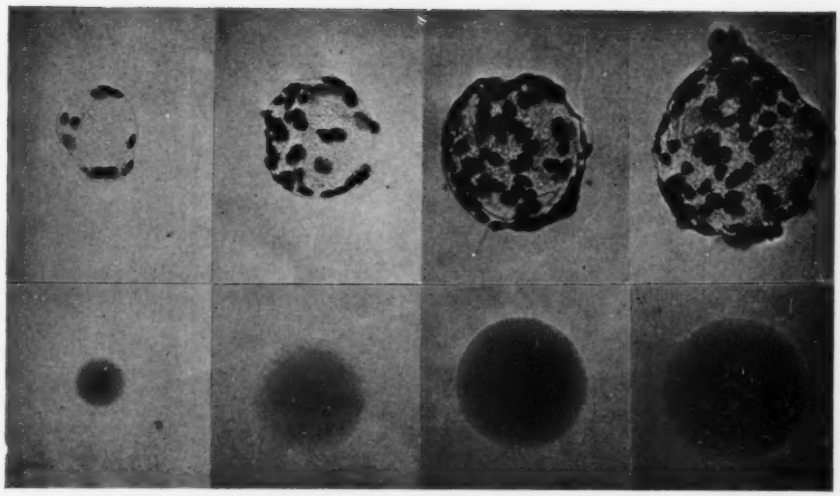

FIG. 1. Isolated spinal ganglion cell bodies with their capsules of satellite cells stained with methylene blue after the extraction of RNA. The RNA extracts, photographed at 257 mμ, as used for the quantitative determinations are shown below the corresponding systems. Magnification: cells ×660, extracts ×133.

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indicated that the capsule occupies about the same percentage volume (33 per cent) for different system sizes. If the degree of submicroscopical folding is unrelated to cell body size a linear regression will consequently exist for the RNA on the surface area of the cell body as well. In any case there is a linear regression of RNA on the square of the third root of the cell body volume.

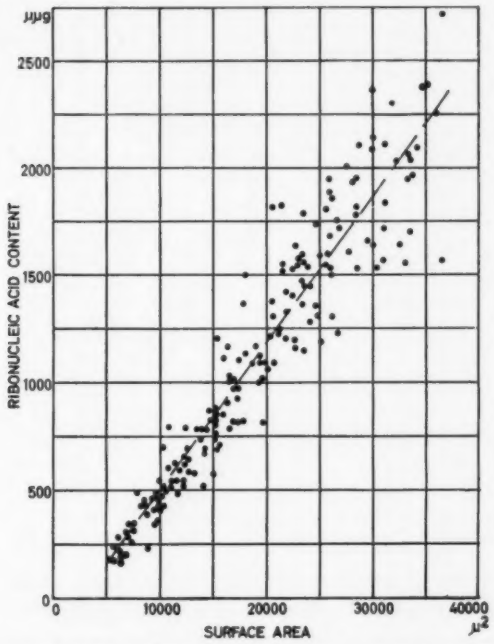

FIG. 2. Values on the RNA content of isolated systems plotted against the corresponding values on surface area.

Measurements of nuclear volumes were made in free-dissected cell bodies as well as systems. The values have been plotted against surface area values for the corresponding cell body or system in Figs. 3 and 4 and regression lines have been constructed graphically. It is evident that the graphs agree well with the findings of BOK (1936) of a proportionality between these two units. The spread of the values is, however, wider than can be explained by the errors in the methods used, showing that other factors also influence the size of the nucleus.

DISCUSSION

It was found during the preliminary part of this work that cell bodies freed from capsules lose part of their RNA content. This implies that there is a risk that, during dissection, capsules would be damaged and some RNA lost, which could pass unnoticed during the preparation. This may occasionally have happened during our experiments. Connective tissue which could not always be completely removed was another source of error. The spread of the values in the RNA graph is, however, not greater than can be explained by these factors together with the random errors of the methods used. They show a distinct linear distribution. Consequently it seems that a strict proportionality between the RNA content of the system and its surface area

(minus a small constant fraction) exists. It is likely that this proportionality also exists between the RNA content of the nerve cell body and its membrane area.

With the nuclear volumes the situation is different. The standard deviations of the individual values from the regression line (about ±25 per cent) are here of a

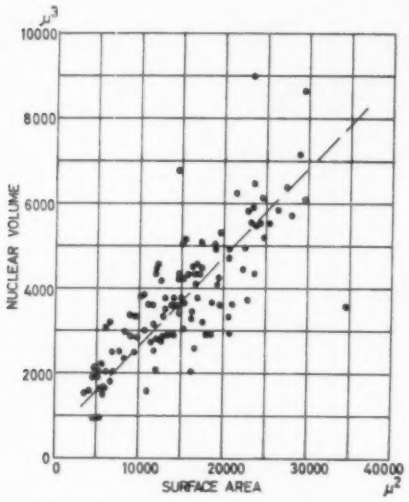

FIG. 3. Values on nuclear volume plotted against the values for surface area in free cell bodies.

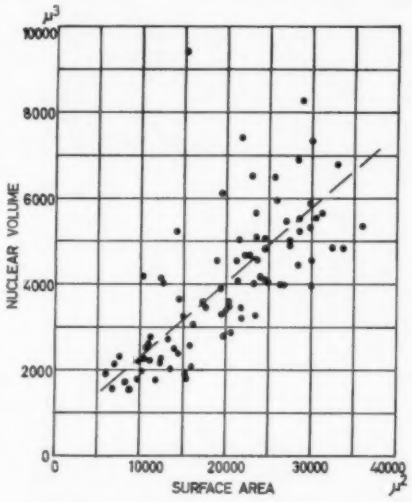

FIG. 4. Values on nuclear volume plotted against the values for surface area in systems.

higher order than can be explained by the standard errors of the methods. (The standard deviation for diameters within nuclei and consequently for volume values based on two of three diameters is ±13 per cent in the present material.) Thus, the area of the cell body surface cannot be the only factor determining nuclear volume. Compared to the graphs on nuclear volumes, the one of RNA values shows a high degree of correlation, which indicates that factors which have influenced the size of the nucleus, other than the surface area, are without any effect on RNA.

Neither the regression line for RNA against cell surface area, nor that for nuclear volume would pass through the origin if extrapolated. These deviations from a strict proportionality act together if one tries to plot RNA against nuclear volume with the result that there will not be any proportionality between these two units. They are, however, correlated to a certain extent so that cells with higher RNA values usually contain bigger nuclei.

Indications for a proportionality between RNA and cell body surface have been found also for motor anterior horn cells (EDSTRÖM, 1956) and for retinal ganglion cells (EDSTRÖM and EICHNER, 1957). There is thus some evidence that the relationship found may be of general application to nerve cells. It seems, however, that it is only valid for true nerve cells, since it has been found that neurosecretory cells do not show it (EDSTRÖM and EICHNER, 1958 and unpublished).

SUMMARY

An investigation has been made of the relationship between the dimensions of free or encapsulated spinal ganglion cells (systems) on one hand, and their RNA

content and the volume of the nerve cell nucleus on the other. Fresh, untreated spinal ganglion cells were used for the purpose. The results show that:

(1) There is a direct proportionality between the surface area of the system (*minus* a relatively small constant fraction) and the RNA content. A proportionality probably also exists between the RNA content and the surface area of the cell body. The deviation of values from the regression line is not higher than can be explained by the errors in the methods used.

(2) There is also a direct proportionality between the nuclear volume (*minus* a relatively small constant fraction) and the membrane area. The deviation of values from the regression line is higher than can be explained by the errors in the methods used.

(3) It is concluded that the surface area is a factor determining the RNA content of these cells and systems and probably also of other kinds of true nerve cells. The results do not exclude the possibility that it is the only factor of importance in this respect, while they clearly show that it is only one of the factors that determine nuclear size.

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QUANTITATIVE DETERMINATION OF RIBONUCLEIC ACID IN THE MICROMICROGRAM RANGE*

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THE lower limit for determination of ribonucleic acid (RNA) according to EDSTRÖM (1953) lies between 100 and 200 $\mu\mu$g. As the majority of the cells contain less than these amounts, it is of interest to lower the limit for quantitative analysis further. The present paper describes a modification of the earlier method allowing determination of RNA in amounts down to 10–20 $\mu\mu$g.

In the original method the extracted RNA was absorbed in cellophane strips which served as cuvettes for the photographic-photometric determination in ultraviolet light. In the present modification RNA is determined instead in solution in drops of regular geometric shape, a change in technique which increases the range of the determination in both directions and which also makes the method simpler to carry out. Some other changes, of a minor nature, have also been introduced.

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METHODS

Cell isolation. Cells or other tissue fragments are isolated either freehand, in which case fresh tissue is used, or with the aid of a micromanipulator when using fixed tissue. Fresh cells are fixed with perchloric acid after isolation and then treated as described by EDSTRÖM and PIGON (1958). Tissue fixation is performed with Carnoy's fluid for 30 to 60 min. The fixed tissue is cut into thick sections (25–100 μ) which are attached to cover slips, 12 × 32 × 0·17 mm in size. After removal of the embedding medium (solid paraffin) the sections are hydrated with a 0·01 N-acetic acid solution and kept under liquid paraffin in an oil chamber (DE FONBRUNE, 1949) made by inverting the cover slip and letting it form the roof of the chamber. The rest of the chamber is formed by a groove, 40 mm long and 3·5 mm deep, across a thick slide 70 × 40 × 6 mm in size. The tissue used is dissected free with one or two glass needles directed by the DE FONBRUNE micromanipulator. Isolated cells are placed in water-free glycerol if determinations of their volume by three-dimensional reconstructions are desired (EDSTRÖM, 1953). They are then washed in 0·01 N-acetic acid, after which they are ready for extraction.

Extraction procedure. The extractions are performed under liquid paraffin. The cover glass forms the roof of one part of the oil chamber, the other part, which is free from paraffin, is covered by a quartz slide. The extractions are carried out with the aid of a micropipette, the diameter of which is determined by the size of the cells. For big nerve cells a diameter of 10–15 μ is suitable. Each cell is extracted three times at room temperature (about 20°C) with volumes of enzyme solution four to five times larger than that of the cell. Before the extractions are begun, the volumes of enzyme solution to be used are placed separately on the cover slip near

* The expenses for the work were defrayed by a grant from the Swedish Cancer Society.

the cells, each of which is extracted with the three solutions one after the other over a period of 1 hr. The average incubation time is thus 20 min. When the RNA has been digested, the fluid is sucked up by means of the micropipette and brought over to the part of the chamber which is free from liquid paraffin. Here the tip of the pipette is put against the quartz glass surface and a slight positive pressure is applied with the aid of a two-way syringe connected to the pipette. The extract then slowly flows out, volatile components of it evaporating meanwhile. The digested RNA is left on the surface in the form of a small spot. After an extract has been transferred to the quartz glass and evaporated to dryness there, the pipette is washed with the next volume of enzyme solution to be used for the same cell. The three extracts are evaporated as close to each other on the quartz glass as possible.

Extraction fluid. Since the volumes of fluid used for extracting RNA have to be evaporated before the extracted RNA can be dissolved for photography, it is advantageous to use an extraction fluid which contains the smallest possible amounts of non-volatile compounds. However, a certain concentration of electrolytes is necessary to extract RNA quantitatively. A solution of Worthington's crystalline ribonuclease is used at a concentration of 0·4 mg per ml 0·2 N-ammonium bicarbonate-acetate buffer, the electrolytes of which evaporate on drying. This buffer is made by adjusting a 0·2 N-solution of ammonium bicarbonate with acetic acid to a pH of 7·6. It is kept cold. Before use the pH is tested and, if necessary, corrected, since it slowly turns alkaline.

The enzyme is prepared according to the McDONALD (1948) modification of the KUNITZ (1940) method. It is stated to be free from protease activity and was found to be without effect on Feulgen stainability.

Quartz slide. A quartz slide, transparent at 257 mμ and 24 × 32 × 0·5 mm in size is washed with hot water and detergent, and rinsed in hot running tap water. It is dried with a linen cloth and placed in chloroform (c.p.) in which it should stay for at least 2 days before use. Before using in the chamber, the slides are marked with ink as a guide for later localization of extracts. After all the cells have been extracted and the extracts transferred to the quartz slide, the second part of the chamber is filled with liquid paraffin. Volumes of a phosphate buffer containing glycerol, roughly adjusted to the amount of RNA expected, are taken with a silicon-treated micropipette of small diameter (3–5 μ) from a supply drop in the chamber and blown out onto the RNA spots. Round drops are thus formed. The quartz slide is then taken from the chamber. Adhering paraffin is allowed to remain on the surface as a mounting fluid and the slide is photographed without a cover slip in an ultraviolet microscope.

Glycerol buffer. The liquid in which the extracted RNA is dissolved is a solution of glycerol in phosphate buffer made by adding five volumes of glycerol to two of 0·2 N-phosphate buffer, pH 7·6. Glycerol is used to bring the refractive index of the RNA-containing drops close to that of the liquid paraffin.

To avoid any significant error in the determinations of RNA, the optical density of the buffer at 257 mμ must not differ greatly from that of the liquid paraffin. In the present work the buffer used had a $D_{1\text{ cm}}^{257\text{ m}\mu}$ of 0·5 while the corresponding figure for the paraffin was 0·8. The difference in optical density corresponds to about 10 μg of RNA per ml. Since the concentration of RNA in the drops is around 10 mg/ml, no measurable error will be introduced in the determinations under these

conditions. Since the buffer and the paraffin have closely similar optical densities, it is unnecessary to prepare special blanks. It is sufficient that the optical density of the background is recorded.

Photography. The RNA-containing spots are photographed in the Köhler ultraviolet microscope using the 257 mμ line, which is purified by means of a Bäckström filter. A $\times 10$ quartz objective (Zeiss, Jena) N.A. 0·20 and a $\times 10$ quartz eye-piece (Bausch & Lomb) are used. The drops are photographed at magnifications between 100 and 300, depending on the drop diameter. The change in magnification is obtained by varying the length of the bellows. For calibration of the light absorption caused by RNA in the drops, and to enable blackening of the plate to be recorded in terms of optical density, a reference system is included in each photograph. Figure 1 shows a series of RNA extracts taken from nerve cell bodies of different sizes.

The reference system, a rotating disc, cut out on a logarithmic scale, is placed immediately in front of the photographic plate. As the spots are situated in front of the objective, the reference system will not compensate for such errors in the optical path between it and the spots that will change the light absorption of the latter relative to that of the background, unless special adjustments are made. Such a possible error is *glare*, i.e. stray light caused by the lenses between the object and the photographic plate (WALKER, 1953). The effect of glare is rather small for the optical densities used for the RNA determinations (below 0·6), and it can be compensated for by means of the reference system.

The magnitude of glare is determined by means of photographing small opaque objects (e.g. metal powder) and determining the apparent optical density obtained. The amount of light which has caused the depression of the value for optical density from infinity to the measured value is a measure of the extent of glare. If I_0 is the incident light, I the light transmitted through the measured area and I_G the light falling on the plate, caused by glare, the value for optical density, D, will be a false lower value of $\log (I_0 + I_G) : (I + I_G)$ instead of $\log I_0 : I$. (As it is not I_0 that is measured, i.e. the light incident to the measured area, but instead the light giving the background blackening of the plate, the expression $I_0 + I_G$ must be used.) By using opaque objects I is made zero and I_G can be determined: $D_{\max} = \log (I_0 + I_G) : I_G$. For the optics used $D_{\max}$ was found to have the value 1·41, giving the result that $I_G = 4{\cdot}1\,\% \times I_0$.

Reference steps in the film emulsion corresponding to equal differences in optical density are obtained by inserting in front of the photographic plate a rotating mask, subdivided into several zones, which have been partly cut away giving light transmitting circumferences of gradually greater angles from the periphery to the centre (THORELL, 1947). The ratio between the angle of the aperture of an outer zone to that of its inner neighbour would be kept constant in a faultless microscope. As the differences in optical density amount to the logarithm of this constant value, there will then between the different steps be a constant difference in the values for optical density. For a reference system used with an optical system which gives rise to glare, the conditions will be somewhat changed. If the ratio of the angle of an outer aperture to that of an adjoining inner one is $A°/B°$, the difference in optical density between these steps will be $\log A°/B°$. For the photographed spots a real value on optical density of $\log A°/B°$ will, because of the glare, be $\log (A° + 4{\cdot}1\,\% \times 360°) : (B° + 4{\cdot}1\,\% \times 360°)$. This error is eliminated by increasing the angles for the open zone circumferences of the reference system so that, at all levels of optical density, it transmits the same amount of 'extra' light that is due to glare. A reference system giving differences in optical density of 0·1505 ($= \log \sqrt{2}$) is used, i.e. the ratio of an outer to an inner angle would have been $\sqrt{2}$ if uncorrected for glare. Expressed in aperture angles the ratio would have been 360°/254·5°. Compensating for glare by adding 4·1 per cent of 360° to both values gives the ratio 374·8/269·3. As, however, the bigger value must be 360° (background blackening) the relationship between the angles will be 360°/258·5°. Thus by increasing the aperture for the first step with 4° the effect of glare on the measured object is neutralized. The calculations of the angles for the subsequent steps are made in a corresponding manner: for step two which should show a value for optical density of 0·3010 ($= \log 2$) or $\log 360°/180°$, 4·1 per cent of 360° is added to both sides, which gives 374·8°/194·8°, which in turn is 360°/187°, etc.

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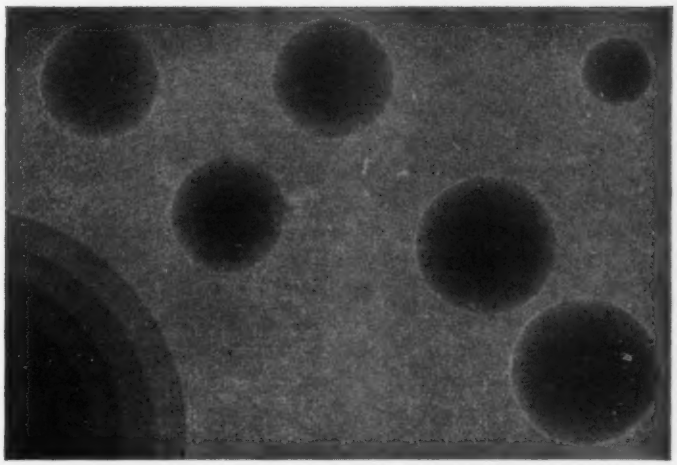

FIG. 1. RNA extracts from nerve cells photographed at 257 mμ together with the reference system. Magnification × 133.

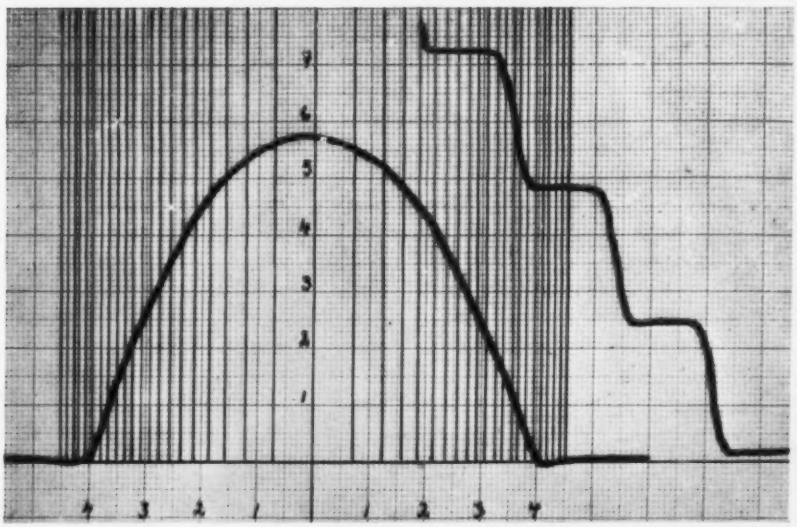

FIG. 3. The photometer track of an RNA extract and the reference system superimposed upon the auxiliary construction used for integrating the optical density.

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The circular disc used for the reference system is of 1 mm thick aluminium and has a radius of 30 mm. The zones have each a depth of 4 mm. The aperture of each zone is distributed in two equal parts opposite to each other in order to balance the disc during rotation (Fig. 2). It rotates at 1500 rev/min.

Kodak O 250 plates, 9 × 12 cm, are used. They are developed in a Kodak D 72 solution and rinsed in water before fixation.

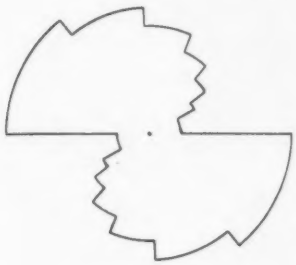

FIG. 2. The reference system used for calibration of the blackening of the photographic plates.

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Photometry. The photographic plates are examined by photometry using a MOLL photometer, model *A*1 (Kipp & Zonen). This instrument has the disadvantage that it does not register values on optical density linearily. It can, however, be put to good use with underexposed plates, where the lower, rounded part of the photographic blackening curve is utilized. In such plates optical density can be recorded reasonably well as a linear function within about five steps of the reference system, i.e. up to optical density values of 0·75.

The photographic print of each drop is examined along two diameters, perpendicular to each other. The recorded curves are superimposed upon each other to facilitate the construction of a mean curve. A track is also taken of the reference system. Each mean curve is then transferred to a transparent paper. In order to make possible rapid integration of the optical densities of the drops, they are treated as if they were subdivided into about ten ring-shaped zones and a central circular area. The areas of all zones are identical with the central one. The mean optical density determined for each zone can thus be added to the others. From the sum of the values for optical density and from the value of the common zone area the amount of RNA can be calculated for each extract. By superimposing the photometer curve over an auxiliary construction (Fig. 3) optical densities in each zone can be found by measuring the height at which the photometer curve intersects its vertical lines. The distances between the lines of the scale and the central line correspond to the mean radii of the zones. The mean radii, i.e. the distances from the centre which divide the zones in two surfaces of equal areas are: $r \times \sqrt{0{\cdot}5}$, $r \times \sqrt{1{\cdot}5}$, $r \times \sqrt{2{\cdot}5}$, $r \times \sqrt{3{\cdot}5}$, etc., where r is the radius of the periphery of the central area. Constructions of different sizes are used for curves of different length so that one always obtains about the same number of subdivisions. As the height in each zone is determined twice, on both sides of the centre, and as each curve is the mean of two diameters it follows that each zone is determined at four points. Thus determinations of about forty points are made for the integration of the optical density of an extract. In spite of this great number of determinations performed by hand the procedure is rapid because of the auxiliary construction.

Calculations. The formula for the calculation of the amount of RNA contained in an extract is:

$$x = \frac{\Sigma h \times f^2 \times s \times 0{\cdot}1505}{a \times 2{\cdot}93}$$

where:

x is the amount of RNA in $\mu\mu$g

Σh is the sum of the mean zone heights (mm)

f is the number of μ to which 1 cm of the photometer curve corresponds, i.e. 10,000 times the inverted value of the magnification in the photometer curve

s is the value (cm^2) of the central circular surface, i.e. the common zone area

a is the height (mm) of each step of the reference system

0·1505 is the difference in units of optical density between each step of the reference system

2·93 is the optical density at 257 mμ of 1 $\mu\mu$g ribonuclease-digested RNA per μ^2 (EDSTRÖM, 1953).

$\frac{f^2 \times s \times 0{\cdot}1505}{2{\cdot}93}$ has a constant value, c, at a given magnification and auxiliary construction. The formula as it is used after the calculation of c is:

$$x = \frac{\Sigma h \,.\, c}{a}$$

RESULTS AND CONCLUSIONS

In order to assess the accuracy of the determination a series of estimations of the recovery of measured amounts of RNA was made. RNA was digested with ribonuclease and dissolved in the glycerol buffer. Its concentration was determined in the Beckman spectrophotometer at 257 mμ. With a silicon-treated micropipette, spheres of this solution were made in the oil chamber and were allowed to fall on a quartz slide placed on the bottom of the chamber, where they formed round spots. The radii of the spheres were measured while still in the upper part of the chamber by means of a ×40 or a ×20 lens and a ×14 measuring eye-piece. In this way spots with known amounts of RNA could be produced. These were photographed in ultraviolet light at 257 mμ. The results of the determinations are shown in Fig. 4. RNA was recovered quantitatively with a stanard error of ±5·3 per cent.

The other departure from the original method which had to be checked was the extraction procedure, as the buffer used in the present modification is different from that in the original method. In order to test whether quantitative removal of RNA occurs from nerve cells when the new, volatile buffer is used, isolated nerve cell bodies, which had been extracted with the latter, were subjected to subsequent extractions with ribonuclease in phosphate buffer, a solution which is known to extract RNA completely from fixed nerve cells (EDSTRÖM, 1953). No more RNA could be extracted from the cells. The completeness of the extraction with the new solution was also ascertained by macrochemical tests. Homogenized spinal ganglia were treated with 1 N-perchloric acid for 7 hr at 25°C, a treatment which also extracts RNA quantitatively from nervous tissue (EDSTRÖM, 1953), after they had been extracted with the new enzyme solution. No measurable amounts of RNA remained for perchloric acid to extract. There is thus good evidence that

the method in its modification allows quantitative extraction and determination of RNA from nerve cells with a standard error of about $\pm 5\%$.

The principle of the method has been used for the quantitative determination of deoxyribonucleic acid (DNA) as well. PIGON and EDSTRÖM (1958) recorded

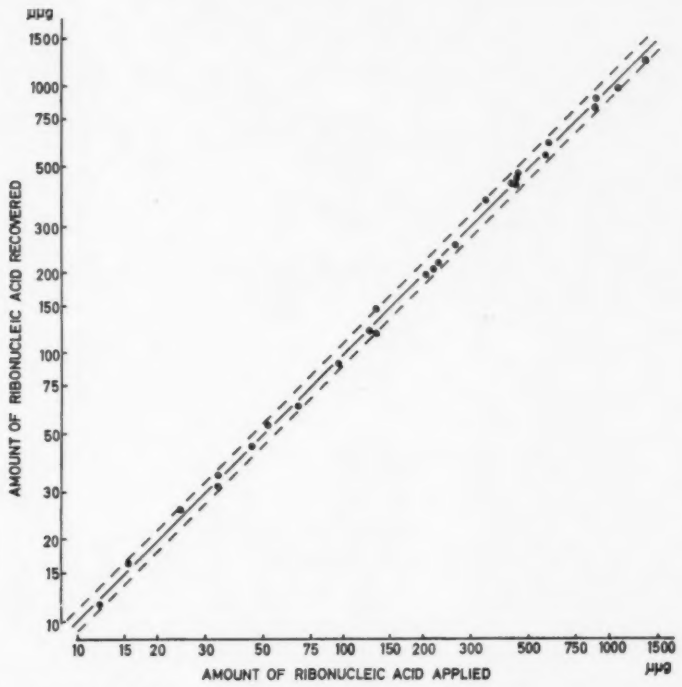

FIG. 4. Graph showing the recovery of measured amounts of RNA. The central of the three diagonal lines corresponds to 100 per cent recovery, the broken side lines to 90 and 110 per cent recoveries.

changes in DNA in individual starving protozoa (*Urostyla*) at different temperatures, but it was found difficult to determine DNA with the same degree of accuracy as RNA.

The sensitivity of the method is illustrated by the fact that it could be used for such small structures as free-dissected nerve cell nucleoli (EDSTRÖM and EICHNER, 1958). The advantage of being able to determine amounts of widely varying magnitudes simultaneously offers special advantages in some investigations (EDSTRÖM and PIGON, 1958). Finally it seems that the method could be adapted without difficulty for autoradiography since the paraffin can be removed from the quartz slides and the spots can be dried.

Acknowledgement—Miss MONA ERIKSSON skilfully assisted throughout this work.

SUMMARY

A method for the quantitative determination of ribonucleic acid (RNA) in mounts down to 10–20 $\mu\mu$g is described. This may be of special value for the

analysis of nervous tissue. The method is a sensitive modification and a simplification of an earlier procedure (EDSTRÖM, 1953). The principle of the method is to determine RNA, which is extracted from free-dissected cells or cell parts, in drops of regular geometric shape by a photographic–photometric procedure in monochromatic ultraviolet light. The standard error is about ± 5 per cent.

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ENZYME ACTIVITIES OF GIANT SQUID AXOPLASM AND AXON SHEATH*

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FROM one giant axon of the squid, *Loligo pealii* can be obtained up to 5 μl of pure axoplasm, i.e. enough for direct chemical analysis. This offers an unusual opportunity to establish the composition of a single histological compartment of nervous tissue. Most chemical information about the nervous system has come from analyses of mixtures of dendrites, axon sheaths, axoplasm, cell bodies, glial elements, and extracellular materials, and is therefore difficult to interpret.

The electrolyte composition of squid axoplasm has been extensively investigated (BEAR and SCHMITT, 1939; STEINBACH and SPIEGELMAN, 1943; KEYNES and LEWIS, 1951; KOECHLIN, 1955). but enzymic or metabolic activities have not been so thoroughly explored. Relative to the sheath, squid axoplasm has been found to be very low in adenosine triphosphatase (ATP-ase) (LIBET, 1948; NACHMANSOHN, 1951–1952) and cholinesterase. On the other hand axoplasm is well supplied with cytochrome oxidase (FOSTER, 1956).

In the present study analyses have been made for ten enzymes, inorganic phosphate, and protein in separated axoplasm and in sheath. All but one of the ten enzymes were found to be present in either axoplasm or sheath, with activities of the same order as those of whole mammalian brain. Some of the kinetic properties of the enzymes were assessed provisionally, in order that analyses might be made at optimal pH and substrate concentration for each enzyme. It was somehow satisfying to find that these enzymes have nearly the same properties in squid axon as they have in rabbit brain.

EXPERIMENTAL

Material. The samples were collected in Cambridge, Massachusetts. The axoplasm from seventy-two axons was separated by extrusion into eleven pools each containing the material from six to ten axons. These were immediately frozen and stored for not over 4 days at $-20°$. The material was then shipped to St. Louis on dry ice and stored at $-80°$ until analyses were complete, a maximum of 40 days. The axon sheaths were collected into three pools and were kept somewhat longer (16 days) at $-20°$. The axoplasm pools had volumes of from 4 to 10 μl each. A total of 40 μl of axoplasm was used for the entire study (approximately 400 determinations). Each pool of axon sheaths weighed from 0·5 to 1 mg per sheath. One pool was used up for the preliminary studies of the properties of squid enzymes.

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† Public Health Service Research Fellow of the National Institute of Neurological Diseases and Blindness.

Methods. A sample of 3 or 5 μl of each axoplasm pool was diluted with 30 or 50 μl of 0·02 M-tris-hydroxymethylaminomethane (tris) buffer pH 7·5. Samples for hexokinase assay were immediately diluted with an equal volume of 0·02 M-phosphate buffer pH 7·7, to stabilize this enzyme. The diluted samples were stored at −80° until all the analyses were completed. Each pool was analysed at least in duplicate for each substance measured.

The enzyme methods were macro-adaptations of micromethods developed for quantitative histochemical studies of brain. The principles of the methods used are as follows (published procedures are indicated): (1) Glucose-6-phosphate dehydrogenase (G6DH) (KUHLMAN and LOWRY, 1956), 6-phosphogluconic dehydrogenase (6PGDH), and *iso*citrate dehydrogenase (ICDH) were assayed by the reduction of TPN^+* by the respective substrates. The TPNH formed was measured by its native fluorescence (LOWRY, ROBERTS, and KAPPHAHN, 1957). Lactic dehydrogenase (LDH), malic dehydrogenase (MDH) (LOWRY, ROBERTS, and CHANG, 1956) and glutamic dehydrogenase (LOWRY, ROBERTS, and LEWIS, 1956) were measured by reduction of the respective keto acids with DPNH. The DPN^+ formed was determined fluorimetrically (LOWRY *et al.*, 1957). Glutamic–aspartic transaminase (LOWRY *et al.*, 1956) was measured in the direction α-ketoglutarate to glutamate in the presence of DPNH and added MDH. The DPN^+ formed was assessed fluorimetrically. Hexokinase was allowed to act on glucose and ATP in the presence of TPN^+ and purified G6DH from hog adrenal glands. The TPNH formed was determined by its native fluorescence. Phosphoglucoisomerase was assessed by the colorimetric (resorcinol) measurement of fructose-6-phosphate formation from glucose-6-phosphate (BUELL, LOWRY, ROBERTS, CHANG and KAPPHAHN, 1958). Glycogen phosphorylase was measured by the inorganic phosphate formed from glucose-1-phosphate in the presence of glycogen primer. The specificity was confirmed by the fact that in the absence of glycogen primer the liberation of inorganic phosphate was decreased by 94 per cent in axoplasm and 92 per cent in sheath. Preformed inorganic phosphate and protein were measured by published procedures (LOWRY, ROBERTS, LEINER, WU and FARR, 1954; LOWRY, ROSEBROUGH, FARR and RANDALL, 1951). All of the enzymes were assayed at 38° under conditions very close to those found to be optimal for squid axon (Table 1). LDH was measured at pH 5·7. 6PGDH was measured at pH 8·2 (tris buffer) with 1 mM-substrate and 0·3 mM-TPN^+.

RESULTS

Kinetics of enzymes of squid axon. There were only a few instances in which a substantial difference was demonstrated between an enzyme of squid axon and the comparable enzyme of rabbit brain (Tables 1, 2; Fig. 1). LDH clearly has a more acid pH optimum in squid than in rabbit (Fig. 1). The pH optimum for transaminase is a little more alkaline than in rabbit. Although the Michaelis constants were not determined with precision, the results indicate that compared to rabbit brain, squid axon hexokinase has a higher K_m for glucose, glycogen phosphorylase has a smaller K_m for glucose-1-phosphate, LDH has a larger K_m for pyruvate, and transaminase has smaller K_m's for both substrates. By omission of adenylic acid, phosphorylase activity was reduced to 65 per cent in the axoplasm and to 27 per cent in sheath.

Enzyme values in axoplasm. The absolute activities of the nine enzymes in axoplasm were found to vary enormously (Table 2). Isomerase, the most active, exceeds ICDH, the least active, by a factor of nearly 700. These activities were measured under nearly optimal conditions, which are not physiological conditions as a rule (Table 1). To fully assess the significance of the activities it will be necessary to calculate the activities under the conditions existing in squid axoplasm and to take into account Michaelis constants and the local concentrations of substrates and coenzymes. Data for such calculation are not now available. However, it may be useful to compare the activities with those of average rabbit brain (Table 3). Comparison has been made on the basis of protein rather than wet weight, since axoplasm

* Abbreviations used include oxidized and reduced diphosphopyridine (triphosphopyridine) nucleotides, DPN^+ (TPN^+) and DPNH (TPNH) respectively, and adenosine triphosphate (ATP).

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TABLE 1.—EFFECTS OF pH AND SUBSTRATE CONCENTRATIONS ON ENZYME ACTIVITIES OF SQUID AXON

The results for hexokinase, isomerase, transaminase and phosphorylase were obtained with axon sheath homogenate. The other results were obtained with axoplasm. Concentrations are recorded as m-moles/l. and are the average values during the incubations, rather than the initial values. Activities are expressed as per centage of the activity under standard conditions.

Hexokinase				*Lactic dehydrogenase*			
pH	Glucose	ATP	Activity	pH	Pyruvate	DPNH	Activity
7·2	7·6	3·4	100	6·6	0·87	0·98	182
7·9	7·6	3·4	98	7·6	0·90	1·01	100
9·0	7·6	3·4	41	7·6	0·28	1·04	51
8·0	1·46	3·5	82	7·6	0·11	1·06	27
8·0	0·31	3·5	50	7·6	0·93	0·29	107
8·0	7·6	0·58	64				
Glucose-6-phosphate dehydrogenase				iso*Citric dehydrogenase*			
pH	G6P	TPN	Activity	pH	*iso*Citrate*	TPN	Activity
8·4	5·0	0·30	100	7·0	0·90	0·65	84
9·4	5·0	0·30	96	8·0	0·90	0·65	100
9·8	5·0	0·30	72	9·2	0·90	0·65	87
9·4	0·82	0·30	69				
9·4	0·15	0·31	39				
9·4	5·1	0·07	88				
Isomerase				*Malic dehydrogenase*			
pH	G6P		Activity	pH	Oxalacetate	DPNH	Activity
7·3	88		90	7·6	1·80	1·00	60
8·0	88		99	8·5	1·80	1·00	100
9·1	88		100	9·3	1·80	1·00	114
				8·7	0·72	1·00	95
				8·7	0·21	1·02	94
				8·7	2·12	0·32	91
Phosphorylase				*Glutamic-aspartic transaminase*			
pH	G1P	AMP†	Activity	pH	α ketoglutarate	Aspartate	Activity
6·2	25	0·80	169	7·1	8·2	41	84
6·8	25	0·80	100	7·9	8·2	41	100
7·4	25	0·80	52	8·4	8·2	41	100
6·7	7	0·80	91	7·9	0·76	41	80
6·8	3·2	0·80	65	7·9	8·3	4·7	80
6·8	25	0·22	79	7·9	8·4	0·45	43
6·8	25	0	65				

* Based on assay of *iso*citrate for fraction oxidizable by TPN and ICDH.
† Adenylic acid.

TABLE 2.—ANALYSES OF ELEVEN SAMPLES OF SQUID AXOPLASM AND TWO SAMPLES OF SQUID AXON SHEATH

All enzyme activities are expressed as m-moles per kg wet weight per hr	Axoplasm	Sheath		
		Sample A	Sample B	Average collagen-free†
Hexokinase	147 ± 7*	32	27	54
Glucose-6-phosphate dehydrogenase	68 ± 1	27	34	56
6-Phosphogluconic dehydrogenase	57 ± 3	12	13	23
Phosphoglucoisomerase	8050 ± 380	340	60	360
Glycogen phosphorylase	86 ± 5	8	4	11
Lactic dehydrogenase	202 ± 21	49	65	104
*iso*Citric dehydrogenase	12 ± 1	68	90	144
Malic dehydrogenase	3300 ± 40	2460	2390	4400
Glutamic-aspartic transaminase	914 ± 19	475	163	580
Inorganic P (m-moles/l.)	12 ± 0·3	8	8	15
Protein-(mg. %)	3240 ± 120	3070‡	3270‡	

* (S.E.M.)
† Corrected for 45 per cent collagen content, see text.
‡ Protein soluble in 0·05 N-NaOH.

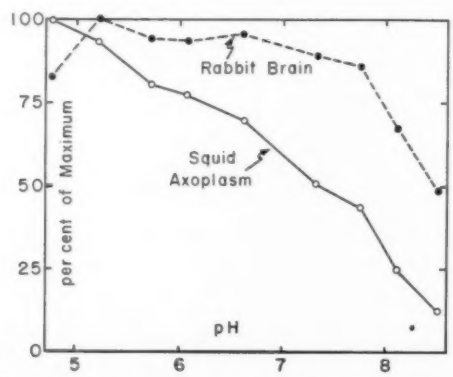

FIG. 1.—Relation of lactic dehydrogenase activity to pH in squid axoplasm and rabbit brain homogenate. A single buffer mixture was used which contained sodium acetate, dipotassium phosphate and tris-hydroxymethylaminomethane, each at a concentration of 0·033 M.

contains only about 3 per cent protein (compared to about 10 per cent for whole brain).

In axoplasm, hexokinase has half the activity of that in the average rabbit brain (hereafter called brain). Two of the three enzymes which use the product of hexokinase, G6DH and isomerase, are about 25 per cent more active than in brain. The activity of 6PGDH confirms the significance of the value for G6DH. Phosphorylase is only half as active as in brain, but since the K_m for phosphorylase is smaller than in brain (Table 1) axoplasm may be at least as active as brain in glycogen synthesis at the low substrate levels likely to be present. Thus, if the enzyme activities are good indices, all three major pathways originating with glucose-6-phosphate are as active

TABLE 3.—COMPARISON OF SQUID AXON AND WHOLE RABBIT BRAIN IN REGARD TO ACTIVITIES AND PROPERTIES OF NINE ENZYMES

Enzyme activities of Table 2 have been recalculated as moles substrate converted per kg of protein per hr. Total protein is used as the basis in the case of axoplasm and rabbit brain, whereas for sheaths the values are based on the protein of the non-collagenous component (see text). Values for K_m are given as m-moles/l.

	Enzyme activities			pH optima		Michaelis constants		
	Axoplasm	Sheath	Rabbit brain	Squid	Rabbit	Substrate	Squid	Rabbit
Hexokinase	4·52	1·80	9·0	7·2–8	8	glucose	0·31	<0·08
						ATP	0·47	0·43
Glucose-6-phosphate dehydrogenase	2·11	1·86	1·5	8·4–9·4	8·0–9·4	G6P	0·18	0·24
						TPN	0·01	0·003
6-Phosphogluconic dehydrogenase	1·76	0·77	1·5		8·2	6PG		<0·13
						TPN		0·002
Phosphoglucoisomerase	249	12	200	8–9	8	G6P	<4	<4
Glycogen phosphorylase	2·66	0·37	5·5	6·2	6·2	G1P	2·2	7·2
Lactic dehydrogenase	6·11	3·5	60	4·5–5	5·2–7·4	pyruvate	0·64	0·15
						DPNH	<0·03	0·01
*iso*Citric dehydrogenase	0·36	4·8	10	8	8	isocitrate	<0·02	<0·02
Malic dehydrogenase	103	147	400	8·5–9	8·7	oxalacetate	0·052	0·046
						DPNH	0·045	0·023
Glutamic–aspartic transaminase	28·3	19	60	8–8·4	7·7	α-keto-glutarate	<0·2	0·59
						aspartate	0·5	2·6

in axoplasm, relative to protein, as in brain. LDH, in contrast, is very low compared to brain; this could mean that glycolysis is not very active. However, the absolute LDH activity is nevertheless so great that glycolysis could be very rapid and a great deal would depend on the level of DPNH. ICDH activity is less than 4 per cent of that in brain and consequently the citric acid cycle as a whole cannot be very active. The ability to oxidize malate is however rather great, although less than in brain. Transaminase, which parallels MDH in retinal layers (LOWRY *et al.*) and in other parts of the brain, is present in roughly the same proportion to MDH in axoplasm as in rabbit brain. Glutamic dehydrogenase, is either absent or less than 5 per cent as active as in brain.

Axon sheath. The sheath values of Table 2 (last column) have been corrected for collagen, presumably inert, which has been found to constitute 45 per cent of the dry weight.* The non-collagenous component of the sheath in turn has been

* The wet weight of cellular material in the sheath was determined from the values for total protein by estimation of the collagen content of the sheath. The latter was performed by Dr. G. DEFFNER by determining the hydroxyproline content by the method of NEUMAN and LOGAN (NEUMAN and LOGAN, 1950) and by assuming that squid collagen has a hydroxyproline content of 10 per cent. Also involved was appropriate allowance for the limited solubility of collagen in 0·05 N-NaOH. It should be pointed out also that a portion of this cellular material in the sheath is represented by fibrocytes rather than by Schwann cells.

estimated to contain 3 per cent protein, and this value has been used in calculating the sheath values of Table 3.

The enzyme activities in axon sheath are in general lower than in axoplasm. Possibly hexokinase can be used as a valid basis of reference for the other enzymes, since it catalyses an obligatory step in glucose utilization. Relative to hexokinase the sheath samples are thirty-five times richer in ICDH and twice as rich in G6DH. On the other hand the isomerase and phosphorylase activities are so low that they could well be attributed to a little residual axoplasm left in the sheath. (As in the case of axoplasm, no glutamic dehydrogenase was found.)

It seems probable, therefore, that the sheath cells depend more than does axoplasm on oxidative processes, particularly those of the citric acid cycle. This seems reasonable since any oxygen reaching axoplasm would have to pass through the sheath.

The isomerase values were so low that the analytical methods might have been suspected. Measurement is based on the fructose phosphate formed from glucose-6-phosphate. However, as a check, the disappearance of fructose-6-phosphate was measured and activity in this direction was also found to be very low. Furthermore, a mixture of axoplasm and sheath homogenate gave the expected summation of activities in both directions. Therefore isomerase activity in these sheath samples seems truly to have been low at the time analysed. This low value together with the low value for lactic dehydrogenase raises the question of possible absence of the Embden–Meyerhof pathway in axon sheath.

DISCUSSION

Whether it is safe to generalize about axoplasm from the data presented for the giant squid axon is open to question. The layer of nearly naked axons from the outer portion of the outer reticular layer of mammalian retina has been analysed for most of the enzymes measured here (LOWRY *et al.*, 1956).* The activities in some respects resemble those of squid axoplasm. Thus this layer is exceedingly rich in G6DH and isomerase, it is very low in glutamic dehydrogenase and it contains more ICDH than any other retinal layer, although the absolute activity is not remarkable. On the other hand LDH is very active (100 times that of squid axoplasm) and hexokinase is very low. The retina of course is rather specialized and may not make a fair comparison.

Myelinated white tracts resemble squid axoplasm in one respect: they are quite rich in G6DH (BUELL *et al.*, 1958). This enzyme may not be located predominantly in the axoplasm however, since among various tracts the activity parallels the lipid content, i.e. it is probably associated chiefly with the myelin or the glia.†

The dissociation of ICDH and MDH in the squid axoplasm, and the association of MDH and transaminase constitute further evidence for the existence in the nervous system of a subcycle consisting of:

(1) oxalacetate + glutamate → α-ketoglutarate + aspartate.

(2) α-ketoglutarate → oxalacetate + CO_2. i.e. a net conversion:

(3) glutamate → aspartate + CO_2.

* LOWRY, ROBERTS and CRAWFORD, to be published.
† MCDOUGAL, to be published.

SUMMARY

(1) The axoplasm and sheath of the squid giant axon have been analysed separately for ten enzymes.

(2) Axoplasm is well equipped with hexokinase and with enzymes of the three major pathways originating with glucose-6-phosphate. It is very low in *iso*citric dehydrogenase and is therefore presumed to be relatively deficient in the citric acid cycle although the presence of substantial quantities of malic dehydrogenase and glutamic–aspartic transaminase suggest a good capacity for conversion of glutamate to aspartate plus CO_2. Lactic dehydrogenase is relatively low in activity.

(3) The values for axon sheath (chiefly satellite cells) are harder to interpret but, judging from the ratio of enzyme activities to hexokinase, the sheath cells depend much more than the axoplasm on the citric acid cycle.

(4) Glutamic dehydrogenase, if present, is less than 5 per cent as abundant in axoplasm as in average rabbit brain based on protein. It was not demonstrated in the sheath.

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A METHOD FOR MULTIPLE ELECTROLYTE ANALYSES ON SMALL SAMPLES OF NERVOUS TISSUE*

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THIS paper describes a system of analysis designed to measure all of the major inorganic electrolytes—Na, K, Ca, Mg, Cl, and P—in a single small sample of nervous tissue.

Because of the interrelationships between the movements of the different electrolytes in living tissue, it is important to be able easily to measure a number of them in the same specimen. At the same time, the multiplicity of the determinations must not increase unduly the size of the sample required. The advantage of limiting the size of the specimen becomes evident in the analysis of small tissues such as retinas and sympathetic ganglia and in the discrete sampling of a heterogeneous organ such as brain. In the latter instance, each significant reduction in sample size opens new opportunities for correlations between chemistry and the function of successively smaller structural units. The potentialities of this approach, as applied particularly to organic compounds in brain, have been remarkably demonstrated by the work of LOWRY and his associates (e.g. LOWRY, ROBERTS and CHANG, 1956).

Methods have been described for the analysis, in biological materials, of small amounts of Na and K (KEYNES and LEWIS, 1951; SOLOMON and CATON, 1955) and of Cl and P (LOWRY, ROBERTS, LEINER, WU and FARR, 1954*a*). Equally satisfactory microanalyses have not been available for Mg and Ca, though GLICK, FREIER and OCHS (1957) have recently published a method for measuring Mg in mμmole amounts using a microscope colorimeter, and a fluorescent indicator for Ca of considerable promise has been synthesized (WALLACH, to be published).

A number of systems for the simultaneous analysis of four or more electrolytes are described in the literature (CULLEN, WILKINS and HARRISON, 1933; YANNET and DARROW, 1938; EICHELBERGER and RICHTER, 1944; LOWRY, HASTINGS, MCCAY and BROWN, 1946; LILLIENTHAL *et al.*, 1950; COTLOVE, HOLLIDAY, SCHWARTZ and WALLACE, 1951). The total size of the specimen required for these analyses varies between 0·3 and 19 g. Different methods of extracting, digesting or ashing the tissue are used prior to the final analyses for the different types of electrolytes; and, since these preparatory steps are not interchangeable, the several analyses require either partitioning of the tissue in its original state or after it has been minced, dried and ground, or homogenized. Partitioning the fresh tissue creates difficulties in interpreting the results if the specimen is not entirely homogeneous. Mincing, grinding, or homogenizing are difficult to perform quantitatively on a microscale and may increase the

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hazard of contamination. They present particular problems when applied to a tissue of high lipid content such as brain. Relating the aliquots to the original sample or establishing some common reference base may involve additional laboratory procedure and represent an added source of error.

In the analytical system to be described, a single preparatory procedure, a wet ashing, was used, which permitted the subsequent analysis of all six of the electrolytes. The techniques used in handling the tissue and in the individual analyses were sufficiently economical to permit all of the determinations to be made on a single 18 mg (wet weight) sample. Conventional type pipettes, cuvettes, and test tubes were used throughout. (A significant further reduction in the size of the specimen may be possible by the application of current microvolumetric techniques.) Of the specific analyses, those for Na, K, Cl, and P were adapted with only minor changes from procedures already published by others. The analyses for Ca and Mg, while similar in principle to previously published methods, embodied significant modifications designed to make them both more sensitive and more reliable. In addition to a specific analysis for Mg and one for Ca, the sum of these ions was measured with an EDTA titration which served as an internal check on the accuracy of the other two analyses.

The accuracy and reproducibility of the complete system were tested by performing replicate analyses on aliquots of a salt solution of known composition, on aliquots of a rabbit brain homogenate, and on aliquots of a mixture of the known salts and the homogenate. Standard deviations and percentage recoveries have been calculated. Results on the brain have been compared with results reported in the literature.

METHODS

The sample to be analysed (from 15 to 35 mg wet weight) was placed in a small (15 × 60 mm) Vycor tube with a plastic stopper and its wet weight determined. It was then dried in an oven at 105°C until there was no further weight loss and reweighed to determine dry weight and total water. The specimen was then ashed.

The wet ashing. Ashing was performed in order to free P for analysis, to eliminate interference by organic compounds in the subsequent analytical reactions, and to insure the release of the divalent cations from combination with organic molecules within the tissue. The several analyses to be performed imposed four general limitations on the ashing procedure: (1) none of the substances to be analysed could be present in the ashing reagents (preferably not even as contaminants); (2) the temperature during the ashing procedure must be low enough to avoid volatilization of P or formation of pyrophosphates; (3) large amounts of acids or other reagents could not remain at the end of the ashing to interfere with the subsequent microanalyses for Ca and Mg; and (4) provision must be made to prevent loss of Cl as HCl.

To meet these conditions, ashing was carried out at relatively low temperatures using 30% H_2O_2 and distilled, concentrated HNO_3. Before beginning the ashing, a known amount of Ag in the form of $AgNO_3$, somewhat in excess of the Cl present, was added to the sample. This served to retain the Cl and was the first step in its subsequent analysis. At the end, the reagents were volatilized leaving a dry ash.

The tubes were placed in shallow holes in a heating block set on top of a small hot-plate whose temperature could be accurately controlled.* The temperature at the bottom of the tubes was adjusted according to the rate of the reaction, but was never more than 150°C. The upper part of the tubes remained cool and their tops were covered with loose-fitting Teflon plugs to cause refluxing. The H_2O_2 and HNO_3 were added separately, a drop at a time. When a drop of H_2O_2 or HNO_3 had been used up to the extent that it no longer underwent visible reaction with the tissue, it was removed by

* Recently, a modification of the heating technique has been adopted. The tubes are placed through holes in aluminium reflectors, and the bottoms are heated with 250 W infrared bulbs controlled by a variable transformer.

drawing a stream of air through the tube; a fresh drop was then added, and refluxing resumed. This prevented an accumulative dilution of the digestants by water and also prevented the rapid and relatively ineffectual decomposition of the H_2O_2 which would have occurred at these temperatures in the presence of the concentrated acid. Furthermore, the digestion of the lipids was apparently hastened by allowing them to dry on the Vycor surface between exposures to the reagents.

When used in this manner, two drops of concentrated HNO_3, followed by two drops of 30% H_2O_2, and then one more drop of HNO_3 were sufficient to reduce 18 mg of brain to a white ash. The entire procedure took about 4 hr. Eight specimens could be digested simultaneously. The operator's continuous attention was not required, though careful observation was needed when reagents were first added as too much heat at this time caused violent reaction with splattering.

The possibility of loss of electrolytes through splatter or entrainment was investigated by determining the recovery in the ash of a known amount of Li, added as Li_2SO_4, to a sample of brain before beginning the digestion. In four such tests, recoveries ranged between 99 per cent and 102 per cent. The completeness of the digestion was estimated in two ways:

(1) The weight of the ash remaining was compared with the weight expected from the amounts of the electrolytes found by analysis—any extra weight presumably representing residual traces of organic material. In four determinations, on aliquots of a brain homogenate, the weight of the ash varied between 104 and 110 μg/mg of dry weight, averaging 107 μg/mg. The total weight of the salts found by analysis was 94 μg/mg of dry weight. Thus, the organic material left after ashing was calculated to be, not more than 13 μg/mg of dry weight, or 1·3 per cent of the original dry tissue.

(2) The values for P determined on tissue ashed by our method were compared with values determined on replicate aliquots of tissue which had undergone a more conventional $H_2SO_4/HClO_4$ ashing as described by LOWRY *et al.* (1954*a*). In six samples of brain, ashed by conventional methods, P averaged 0·527 μmoles/mg of dry weight with a standard error of $\pm$0·004 μmoles/mg. In six samples taken from the same homogenate and ashed by our method, P averaged 0·524 μmoles/mg with a standard error of $\pm$0·005 μmoles/mg, or within 1 per cent of the former value.

Treatment with resin and sampling. The ash remaining after the digestion was dissolved in 2 ml of 0·01 N-HNO_3. Aliquots (0·1 ml) were taken for P analysis and Ag analysis. Of the remaining 1·8 ml, 1·7 ml was put through a small column containing 0·7 ml of Dowex 2 resin in the Cl cycle, and it was rinsed through with 1·0 ml of 0·01 N-HCl.* The purpose of the resin was to remove phosphate. This was found necessary to avoid interference by the phosphate in the subsequent analyses for Ca and Mg, all of which were performed in alkaline solutions.

The 2·7 ml recovered from the column contained all of the cations (except Ag) as their chlorides in 0·01 N-HCl. It was partitioned as follows: 0·5 ml was used for the Na and K analyses; 0·5 ml for the Mg analysis; 1·0 ml for the Ca analysis; and 0·5 ml for the determination of Ca and Mg by EDTA titration.

Specific Analyses

Cl was determined, according to the method of LOWRY *et al.* (1954*a*) by measuring the free Ag remaining after the addition of a known amount of $AgNO_3$, added in excess of the Cl.

P was measured, also by a method of LOWRY *et al.* (1954*a*), the initial ashing described in the original procedure having been replaced by the HNO_3–H_2O_2 ashing described above.

Na and K were determined using a Perkin–Elmer internal standard flame photometer in which the original atomizing system had been replaced by a microatomizer, atomizing into a hot chamber made according to DUBBS (1952). With these changes a reading could be made with less than 0·5 ml of solution. The possibility of error due to the presence of organic materials or of varying amounts of PO_4^{3-} or SO_4^{2-} (MARGOSHES and VALLEE, 1955) was eliminated by the ashing and the use of the exchange column.

Magnesium. Mg was determined colorimetrically using titan yellow. In this reaction a pink lake is formed when the dye is added to the Mg and the solution made alkaline. Its dispersion is maintained by a stabilizer, such as polyvinyl alcohol. Our analysis represented a compilation of features described in a number of previously published methods (ORANGE and RHEIN, 1951; KUNKEL, PEARSON and SCHWEIGERT, 1947; GILLAM, 1941; and GARNER, 1946) including some modifications of our own.

* With 200–400 mesh resin, the surface tension of water was sufficient to stop the meniscus when it reached the surface of the resin. Thus, the column delivered precisely whatever volume of fluid was added at the top, and no stopcock or clamp was required.

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We found that the amount of colour developed in the analysis was affected by even small changes in almost every reagent used. The effects of systematically varying the different constituents of the reaction mixture were studied. A level that appeared optimal was selected for each constituent and maintained within close tolerances in the subsequent analyses.

Two reagent mixtures were used. The first reagent mixture was made up by adding an intermediate dilution of a stock solution of the dye to a solution of polyvinyl alcohol and $CaCl_2$ to give

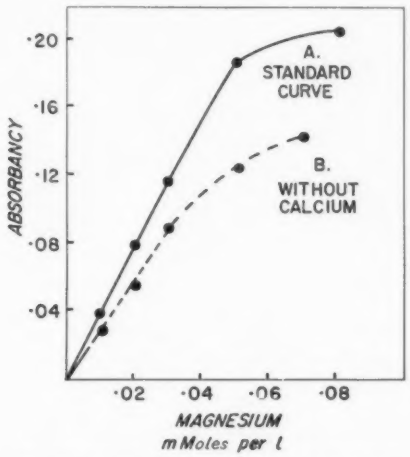

FIG. 1.—Standard curve for magnesium and similar curve obtained without the addition of 1·67 m-moles/l. of calcium.

a final mixture containing 0·002 per cent titan yellow, 0·5 per cent polyvinyl alcohol and 5 m-moles/l. $CaCl_2$. The stock solution of titan yellow, 0·5 per cent in water, was stable for at least 3 months when stored in plastic in the cold. The second reagent mixture contained 0·2 N-NaOH and 0·01 M-NaCN.

Blanks and bracketing standards were always developed and read together with the unknowns. The blank, standard or unknown (0·5 ml) contained in a solution of 0·01 N-HCl, was placed in a Pyrex Beckman cuvette. The first reagent mixture (0·5 ml) was added and mixed by swirling; the second reagent mixture (0·5 ml) was then added and mixed. The cuvettes were capped with Parafilm, allowed to stand for a minimum of 20 min and read at 545 mμ. The final volume was only 1·5 ml. It was found that this was sufficient for an accurate reading in either the Beckman Model DU or the Beckman Model B spectrophotometers, providing a filler about 8 mm high was placed in the cuvette holder under the cuvette to raise the column of fluid up to the level of the light path. The colour was stable for about 2 hr after the preliminary 20 min period, if the tubes were kept in a dim light.

The final concentrations present in the cuvettes were: Mg 0·01–0·03 m-moles/l.; titan yellow 0·0096 m-moles/l. (or 0·0067 g/l.); polyvinyl alcohol 1·67 g/l.; Ca 1·67 m-moles/l.; CN^- 3·33 m-moles/l.; OH^- 0·0633 N.

Curve *A* in Fig. 1 shows a standard curve extended into the region where dye concentration becomes limiting. With a final dye concentration of 0·0096 m-moles/l. the absorption is linear up to final concentrations of Mg of about 0·04 m-moles/l. (or, surprisingly, to Mg : dye ratios of 4 : 1). The molar extinction coefficient is about 4000.

A number of extraneous factors were found to interfere with the reaction. Sunlight caused rapid fading of the developed colour and had to be avoided. A substance, or substances, present in concentrated HNO_3 and extracted from soft glass or Pyrex by hot acid solutions was found to impair colour development and produce fading. It was eliminated by using distilled HNO_3 and Vycor containers for the ashing procedure. Ag, $PO_4^{3(-)}$, and Fe were found to impair colour development and, in some cases to precipitate the lake. The first two were removed by the column. Possible effects due to the presence of traces of Fe were eliminated by adding cyanide to the reaction mixture.

Ca, present in small amounts, increased the colour developed in the reaction; but, at levels

considerably above those expected from tissue, even large variations produced no further effects. Ca was therefore added to the reagent mixture to eliminate it as a variable. Its effect in enhancing the colour can be seen by comparing the absorptions obtained without Ca, shown in curve B (Fig. 1) with those of the usual standards, curve *A*. A further advantage resulting from the addition of Ca was an increase in the stability of the colour developed.

Calcium. Ca was measured colorimetrically using murexide (ammonium purpurate) in a modification of the method of WILLIAMS and MOSER (1953). Ca complexes with murexide in alkaline solutions, producing a change in the colour of the dye from purple to pink. The maximum absorption of the Ca–dye complex is at 506 mμ which corresponds closely to the wavelength at which an isobestic point exists for ammonium purpurate solutions containing up to 0·2 m-moles/l. of Mg. As a result of this fortunate coincidence, Mg does not interfere with the determinations.

Because of the rather low association constant between Ca and murexide (about $10^{-4.9}$ at pH 12) relatively high concentrations of dye were required to obtain near-complete complexing of the small amounts of Ca present in our aliquots. This resulted in correspondingly high blanks, and in order to obtain accurate readings, unusual care had to be taken to provide precisely the same concentration of dye in all of the cuvettes. It was found that the tendency of the dye to fade when in its alkaline form was almost completely abolished at 0°C. Accordingly, solutions were prepared in the cold and brought to room temperature just before reading. It was found undesirable to read the cuvettes while they were cold since the absorption of the dye was significantly affected by changes in the temperature of the solution, and uniform temperatures in the cuvettes were not easily maintained if they were much below the ambient temperature. This latter effect of temperature was apparently distinct from the irreversible fading which occurred in the warm.

Samples (1 ml) of the blank, standard, or unknown solutions (in 0·01 N-HCl as usual) were delivered into Pyrex Beckman cuvettes. The cuvettes were then cooled in ice water and 0·5 ml of the ice-cold reagent mixture containing NaOH and murexide were added. To insure precise reproducibility in the amounts of dye delivered, this addition was made with a 0·5 ml constriction pipette, the tip of which had been dipped into molten polyethylene to make it hydrophobic. The standards and unknowns were transferred together from the ice bath to a warmer water bath to bring their temperature quickly up to that of the room air. They were then dried on the outside and read immediately in a Beckman Model DU spectrophotometer at 506 mμ. As in the Mg analysis, fillers were inserted below the cuvettes to permit readings with a final volume of 1·5 ml. The molar extinction coefficient was about 9000, or somewhat less than that obtained by WILLIAMS and MOSER (11,000) using different conditions.

The reagent mixture was made up immediately before use by combining 1 part of a solution containing 0·6 μmoles/ml (18 mg%) of murexide in 70% ethanol with 4 parts of 0·07 N-NaOH. When stored in plastic in the cold, the ethanol solution of murexide was stable for 2½ months. The final concentrations present in the cuvettes were: Ca 0·003–0·013 μmoles/ml : murexide 0·04 μmoles/ml; and OH^- 0·012 N.

Calcium plus magnesium. The sum of Ca and Mg was determined by titration with ethylenediaminetetraacetic acid (EDTA) using Eriochrome Black T as an indicator. This dye was introduced as a metal indicator by SCHWARZENBACH and BIEDERMANN (1948). A review of its uses in EDTA titrations has been recently compiled by BARNARD, BROAD and FLASCHKA (1956, 1957). Our analysis was adapted from the method of ISHII (1952) including features suggested by Dr. NANCY NICHOLS. Under the conditions of the titration, the colour of the dye was primarily dependent on the presence or absence of free Mg ions. The titration therefore measured all ions in the solution with association constants for EDTA equivalent to, or greater than, that of Mg. In addition to Ca and Mg, these would have included small amounts of Fe, Cu and Zn. Fe and Cu were eliminated from the reaction by the presence of relatively high concentrations of CN ions in the solutions being titrated. Zinc is present in brain in amounts equivalent to about 2 per cent of the sum of the Ca and Mg (SHOHL, 1939) and since about two-thirds of this was also shown to be bound by the cyanide, the net contribution of Zn to the titrations was practically negligible.

An unknown solution or standard (0·5 ml) was placed in a small (10 × 35 mm) Pyrex vial. A reagent mixture (0·5 ml) containing the dye, a NH_4OH–NH_4Cl buffer, and NaCN was added. The resulting pink solution was titrated to a blue colour with EDTA, using a syringe microburette (Micro-metric Instrument Co.). The end point was determined by comparison with a control vial which differed from the test vial in that a blank solution replaced the standard or unknown. For

most accurate results, a small amount of extra water was added to the control vial so that its volume was similar to that of the test vial at the end of the titration. Mixing was maintained with a small magnetic flea, using a Mag-mix.

The blank, standards, and unknowns were, as usual, in solutions of 0·01 N-HCl. The reagent mixture was made up by combining one part of a methanol solution containing 0·25% (w/v) Eriochrome Black T and 2·26% hydroxylamine HCl with 100 parts of an aqueous solution containing 0·004 moles/l. of NH_4Cl, 0·11 moles/l. of NH_4OH, and 0·02 moles/l. of NaCN. When kept cold, this mixture could be used for a period of up to 24 hr. The stock methanol solution of dye with hydroxylamine was stable in the cold for at least 10 months. The final mixture when ready for titration had a pH of 10.1–10.2 and contained 0·00125% (w/v) Eriochrome Black T and 0·01 moles/l. of NaCN. The titrating solution was made up from the disodium salt of EDTA to a concentration of 0·0005 M with sufficient NaOH added to bring it, also, to a pH of 10·1–10·2.

Contamination, presumably by traces of extraneous metals, constituted a major problem in the development of this analysis. In most instances the offending substances were not identified, but they were found to be present in the laboratory distilled water, in soft glass, in the cellulose tissues used to wipe the end of the pipettes, and in some of the reagents. Ferric iron was a potential offender, interfering in concentrations as low as 0·03 μmoles/ml. Pyrophosphates caused the titre to be erroneously low, probably by chelating Mg ions; and care had to be taken to avoid their formation in the ashing procedure. Because of these difficulties due to contaminants, a number of the precautionary measures described in the following section were instituted, including distillation of the NH_4OH, substitution of Pyrex for the original soft glass titration vials and the use of Whatman 40 filter paper instead of tissues for wiping pipette tips.

With these precautions, the fading of the dye was markedly retarded, even in alkaline, aqueous solutions; the dye in the unknown solutions was entirely comparable to the dye in the standard solutions; and the end points were sharp and reproducible. The consistency of the analysis is indicated by the results obtained on titrations of a single standard solution carried out over a period of months. The aliquot used contained 0·045 μmoles of Mg and the titre averaged 0·094 ml of the EDTA solution with a standard deviation of less than 0·001 ml.

Avoidance of contamination. Because we were handling small amounts of commonly found ions in relatively large volumes of solution, contamination was an ever-present problem. Almost everything related to the analyses seemed to be a potential source. Glass, even Pyrex, was found to contribute significant amounts of Na and Ca to solutions in contact with it and was replaced wherever possible with plastic or Vycor ware. Polyethylene bottles were used for storing reagents and standards, alkaline solutions being kept in desiccators to avoid accumulation, through the plastic, of CO_2. Pyrex pipettes were used in place of the more common soft glass variety. Acid-washed filter paper was used to wipe pipette tips as other types of cellulose tissues were found to contribute a contaminant that interferred with the Ca and Mg analyses. Washing and rinsing were performed with unusual care; all containers as well as the pipettes being soaked in 2 M-HNO_3 and rinsed with resin-treated water as the last step.

All aqueous solutions were made up with distilled water that had passed through a 'Deminac' mixed-resin cartridge. The HNO_3 used in the digestion was distilled in a Pyrex still at reduced pressures with temperatures not above 25°C. NH_4OH solutions were made up from NH_3 distilled in a polyethylene still. NaOH solutions were made by diluting a saturated solution of NaOH and were immediately stored in plastic containers.

Even with these precautions, blank runs of the entire analytical system revealed Na contamination averaging 0·04 μmoles and Ca contamination of 0·0016 μmoles—or 4 per cent and 6 per cent respectively of the total amounts of these ions present in an average sample.

An excellent discussion of the problems of contamination in metal analysis has been recently published (THIERS, 1957).

EVALUATION OF THE METHODS

The system of analysis described above was tested by using it to measure the electrolytes in aliquots of an homogenate of rabbit brain, in aliquots of a solution of salts of known composition, in mixtures of the brain homogenate and the known salt solution, and in tubes which were initially empty and which were carried through

the entire procedure as blanks. Six consecutive sets of analyses, using the complete system including the wet ashing, were made on each of these four preparations.

The brain homogenate was prepared by homogenizing a whole rabbit brain in about eight times its volume of water using a Lourdes high speed homogenizer. Aliquots of the homogenate (0·16 ml) corresponding to about one-five-hundredth of

TABLE 1.—THE COMPOSITION OF THE SOLUTION OF KNOWN SALTS; ITS PARTITIONING FOR THE SEVEN SPECIFIC ANALYSES; AND THE RESULTS OF THESE ANALYSES. Cl AND SO_4 IONS, ALSO PRESENT IN THE SOLUTION, ARE NOT INCLUDED IN THE TABLE. EXCEPT FOR THE FIGURES GIVEN AS PERCENTAGES, ALL VALUES ARE IN μ-MOLES

	Na	K	Mg	Ca	Ca + Mg	Cl	Ag*	P
Total amount of each electrolyte in a single 0·1 ml sample of the known salt solution	1·00	1·50	0·100	0·025	0·125	1·06	1·50	1·50
Proportions of original sample used in aliquots for the specific analyses	7·87%	7·87%	15·8%	31·6%	15·8%	—	5%	5%
Amount of each electrolyte in the aliquot used for its analysis	0·0787	0·118	0·0158	0·0079	0·0197	—	0·0220	0·0750
Amount found by analysis (average of six determinations)	0·0791	0·1179	0·0152	0·0080	0·0199	—	0·0235	0·0752
S.D.	0·0014	0·0007	0·0006	0·0005	0·0005		0·0005	0·0013

* Added as $AgNO_3$ before subjecting the sample to the ashing procedure. After precipitation of AgCl, only 0·44 μmoles remained as Ag ion, of which 5% (0·022 μmoles) was used for the analysis.

the total brain, were used for the analyses. Each aliquot contained the equivalent of 17·5 mg of wet brain and weighed 3·61 mg when dried. The solution of known salts was so composed as to provide, in a single 0·1 ml aliquot approximately the same amounts of the different electrolytes as were present in the aliquots of the homogenate. Its exact composition is shown in Table 1. The mixtures of the homogenate and known salt solution were made by adding 0·16 ml aliquots of the homogenate to 0·1 ml aliquots of the salt solution.

The results of the analyses on the blank tubes were zero for all of the electrolytes except for Na and Ca. The average amount of the Na blank, per tube, was 0·04 μmoles and that of Ca 0·0016 μmoles. The values for Na and Ca presented below were obtained after subtracting these blanks.

Table 1 shows the proportions of the 0·1 ml sample of the known salt solution which were allocated to each of the seven specific analyses, the amount of the electrolyte in question in each of the final aliquots, and the amounts of each of the electrolytes

found by analysis. The results are the averages of six consecutive determinations. The figures for the standard deviations include variations that may have been introduced during the course of the ashing procedure and the resin treatment as well as those inherent in the final analyses.

TABLE 2.—THE RESULTS OF ANALYSES ON 0·1 ml ALIQUOTS OF THE SOLUTION OF KNOWN SALTS (COMPOSITION SHOWN IN TABLE 1); ON 0·16 ml ALIQUOTS OF THE RABBIT BRAIN HOMOGENATE CONTAINING 3·61 mg DRY WEIGHT OF BRAIN; AND ON MIXTURES OF 0·1 ml OF THE SALT SOLUTION WITH 0·16 ml OF THE BRAIN HOMOGENATE. THE RESULTS ARE THE AVERAGES OF SIX CONSECUTIVE SETS OF DETERMINATIONS ON EACH OF THE THREE PREPARATIONS

Analyses for	Material analysed	Average results (μmoles)	Standard deviation (μmoles)	Recovery of known salts (%)
Na	Known salts (Na = 1·00 μmoles)	1·00	0·02	100
	Rabbit brain	0·89	0·01	
	Brain + salts	1·89	0·03	100
K	Known salts (K = 1·50 μmoles)	1·50	0·01	100
	Rabbit brain	1·57	0·02	
	Brain + salts	3·08	0·07	100·6
Mg	Known salts (Mg = 0·100 μmoles)	0·096	0·003	96
	Rabbit brain	0·098	0·004	
	Brain +salts	0·203	0·007	105
Ca	Known salts (Ca = 0·025 μmoles)	0·025	0·002	100
	Rabbit brain	0·023	0·002	
	Brain + salts	0·051	0·002	112
Ca + Mg	Known salts (Ca + Mg = 0·125 μmoles)	0·126	0·003	100·8
	Rabbit brain	0·127	0·003	
	Brain + salts	0·253	0·004	100·8
Cl	Known salts (Cl = 1·06 μmoles)	1·01	0·01	95
	Rabbit brain	0·67	0·02	
	Brain + salts	1·73	0·02	100
P	Known salts (P = 1·50 μmoles)	1·51	0·02	100·6
	Rabbit brain	1·85	0·06	
	Brain + salts	3·29	0·09	96

In Table 2, the results of the analyses on the solution of known salts are again presented, this time multiplied by the appropriate factors to give the amounts of the electrolytes present in the original 0·1 ml sample. Results, similarly calculated, are presented for the 0·16 ml samples of brain homogenate containing 3·61 mg of dry

TABLE 3.—A COMPARISON OF PREVIOUSLY REPORTED RESULTS OF ELECTROLYTE ANALYSES ON BRAIN WITH THOSE PRESENTED IN THIS PAPER

Type of tissue analysed	Weight of tissue used for the analyses* (g)	Component (m-moles/kg of wet tissue)					
		Na	K	Mg	Ca	Cl	Total P
Cat brain[1] Mean of 17 animals ± S.E.		53·9 ± 0·9	87·5 ± 1·3	—	—	41·3 ± 0·8	86·0 ± 3·1
Rat brain[2] Mean of 46 animals ± S.E.	2+	45·7 ± 0·4	99·3 ± 2·0	6·8 ± 0·5	2·0 ± 0·4	34·6 ± 0·6	106·0 ± 0·5
Dog cerebrum[3] Mean of 12 animals ± S.D.	19	51·0 ± 2·4	95·6 ± 4·7	5·63 ± 0·56	1·07 ± 0·07	36·71 ± 1·05	—
Rabbit grey matter[4] 1 animal		52·1	—	—	—	39·7	—
Rabbit brain[5–9]	—	—	86·4[5]	5·9[6] 5·7[8] 5·6[9]	2·5[5] 1·0[8] 1·7[9]	36·7[6]	123·8[7]
Rabbit brain, Our data 1 animal, 6 replicate analyses ± S.E.	0·0175	51·0 ± 0·33	90·0 ± 0·06	5·59 ± 0·10	1·31 ± 0·04	40·0 ± 0·06	105·8 ± 1·6

* The weight of each specimen used for the set of multiple analyses, when reported.

[1] YANNET and DARROW (1938).
[2] LOWRY *et al.* (1946).
[3] EICHELBERGER *et al.* (1949).
[4] MANERY and HASTINGS (1939).
[5] CALLISON, LANDER and UNDERHILL (1930).
[6] BRODIE, as quoted by MANERY (1954).
[7] LOWRY *et al.* (1954*b*).
[8] GOTO (1922).
[9] ROMENSKI (1935).

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brain and for mixtures of 0·1 ml of the salt solution with 0·16 ml of the brain homogenate. Table 2 shows the standard deviations of the six determinations in each group and the percentage recovery of the known salts, both when they were analysed alone and when they were determined as additions to the homogenate. The standard deviations varied between 0·7 per cent and 3·4 per cent of the average values, for all of the determinations except those for Ca. The standard deviation of 0·002 μmoles in the Ca analysis on rabbit brains was 9 per cent of the total value and made this the group with lowest reproducibility.

The recoveries of the known salts ranged between 95 per cent and 105 per cent, with the exception of the recovery of Ca added to brains for which a value of 112 per cent was calculated. The recovery for the Cl in the known salts of 95 per cent with a standard deviation of only 1 per cent indicated a reproducible loss. This apparently had occurred during the mock ashing in the form of HCl. To see whether it also had occurred during the ashing of the brain we compared the values for the ashed brain with those for replicate aliquots which had been extracted with 0·75 N-HNO_3 (LOWRY and HASTINGS, 1942) instead of ashed and found them also 5 per cent low (0·67 μmoles vs. 0·70 μmoles). The reproducibility of this loss was further indicated by the fact that the difference between the Cl found in the brain plus salt mixture and that in the brain alone came out to be the theoretical 1·06 μmoles. If the conditions of the ashing are kept fairly constant, a small, fixed correction can be reasonably applied.

The values in Table 2 for Ca plus Mg as determined by the EDTA titration are to be compared with the sum of the values for Mg as determined with titan yellow and those for Ca measured with murexide. In the case of the analysis on the brain the result of the EDTA titration (0·127 μmoles) was 0·006 μmoles, or 5 per cent higher than the sum of the two specific analyses (0·121 μmoles).

Table 3 shows the average of our results on the brain samples expressed in terms of millimoles per kilogram of wet weight of brain for purposes of comparison with data reported in the literature. The value for Cl includes the correction indicated above. It is evident that there is considerable variation in the reported values, only part of which can be explained by species differences. Our results do not appear to differ significantly from the average. It should be pointed out that the standard errors calculated for our data represent only those variations inherent in the analytical procedures and do not include two potentially important sources of variation that are included in the standard errors and standard deviations presented from the literature, namely, those resulting from sampling and those resulting from the differences between individual animals. BARNES, GORDON and COPE (1957) have clearly shown the importance of distinguishing between these main sources of variation and have demonstrated how they may be handled statistically.

SUMMARY

A system of analysis is presented by means of which all six of the major inorganic electrolytes can be determined on a single 17·5 mg sample of tissue. The accuracy and reproducibility of the methods have been evaluated by replicate analyses of salt solutions of known composition and of aliquots of an homogenate of rabbit brain. The results obtained on the rabbit brain have been compared with data reported in the literature.

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LIPID BIOSYNTHESIS *IN VITRO* BY ELECTRICALLY STIMULATED RAT SCIATIC NERVES*

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LIPID biosynthesis *in vitro* by peripheral nerves, and presumably the formation of myelin lipids, was shown to undergo wide variations not only in experimental neuropathies (MANNELL and ROSSITER, 1954; MAJNO and KARNOVSKY, 1958*b*) but also physiologically, with relation to age (MAJNO and KARNOVSKY, 1958*a*). It would be interesting to know whether lipid biosynthesis is also affected by the state of excitation of the fibres, i.e. whether the passage of an impulse affects the incorporation of simple labelled building blocks into the nerve lipid. The problem was approached as follows: modified Warburg flasks were constructed, in which a rat sciatic preparation could be suspended between two pairs of electrodes. It was thus possible to stimulate the nerves and to record the ensuing impulses in the course of incubation. Respiration could also be followed. The medium contained one of four isotopically labelled lipid building blocks. When the lipid extracts of stimulated and resting nerves were compared, it was found that two of the substrates showed a greater incorporation into the lipids as a result of stimulation.

MATERIAL AND METHODS

The modified Warburg flasks used in these experiments are shown in Fig. 1. In addition to the neck which fits onto the manometer, they have another vertical socket, 3 mm wider, designed to receive a lucite stopper fitted with two pairs of stainless steel wire electrodes. The problem of passing the wires through the lucite stopper was solved at first by boring small holes through which the electrodes were passed and fixed with various types of cement. None of these stoppers was entirely gas-tight. Excellent plugs were finally obtained as follows: a square measuring about 3 × 3 cm was cut from a lucite plate 5 mm thick. On both faces of the square two parallel grooves were etched, 9 mm apart. The wires were laid in these grooves, and the square was then glued sandwich-wise between two lucite plates 9 mm thick. The result was a rectangular block of lucite in which the wires were firmly embedded, and which could easily be machined into a plug of proper taper. When the latter was inserted into the neck of the flask, the tips of the electrodes were arranged to be 3 mm above the level of the incubating medium (see below). Despite a flask capacity of about 30 ml, which is double that of average Warburg vessels, the accuracy of the respiratory measurements was found to be satisfactory.

The experimental flasks were provided with: main chamber, 5·0 ml of phosphate-buffered Krebs–Ringer solution at pH 7·4 with 10 μmoles/ml of glucose; centre well, 0·2 ml 20% NaOH; side arm, 0·3 ml of Krebs–Ringer solution containing the isotopically-labelled lipid precursors. Four such substances were used: [1-^{14}C]acetate synthesized by a standard method (SAKAMI, EVANS and GURIN, 1947), [1:2-^{14}C]choline obtained from the California Research Foundation, [^{14}C]glucose (uniformly labelled) obtained from the Nuclear Corporation, Chicago, and [^{32}P]orthophosphate,

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obtained from Oak Ridge and used as potassium orthophosphate. The labelled organic substrates, except glucose, were used at a concentration of 3·0 mg per flask. Glucose and phosphate incorporation were studied at the concentration of the Krebs–Ringer medium mentioned above. Each flask contained routinely 0·75 to 3·0 $\times$ 10^6 counts/min.

The rats were males of the Sprague–Dawley strain aged 95 to 110 days and weighing 350 to 450 g. For each experiment, a single rat was killed by decapitation, the sciatic and its tibial branch were bilaterally exposed, covered with a few drops of Krebs–Ringer–phosphate solution, and rapidly excised. In order to minimize trauma only the major clusters of epineurial fat were dissected off, a thorough cleaning being performed after the incubation. Each preparation measured 5·5 to 6·5 cm in length. The nerve was weighed to the nearest 0·2 mg on a torsion balance; the average weight was from 55 to 65 mg. Its proximal end was fastened on to the stimulating electrodes, and the distal end onto the recording electrodes; the nerve was then crushed between the two recording electrodes. When the plug was inserted into the flask, only the middle portion of the nerve, measuring from 2·5 to 3·5 cm, was bathed in the fluid.

Both flasks were placed in the constant temperature bath at 37° C, gassed with pure O_2 for 10 min, and equilibrated for 20 min. The side arms were tipped and the first manometric reading was taken after 10 min. Stimulation was then started in one of the flasks. This was carried out with a Grass S4A stimulator and isolation unit, using a 0·1 msec square-wave pulse. Stimulus strength was maintained at from two to three times maximal for A-α fibres. The frequency was either 60 pulses/sec for 2 hr, or 300 pulses/sec for 3 hr. The action potential was observed throughout the experiment by means of an a.c. amplifier and oscilloscope, and manometric readings were taken every 30 min. The volume of gas generated by electrolysis during the entire experiment was calculated and found to be negligible. This was further verified by running four control experiments in which either 60 or 300 pulses/sec were applied for 3 hr to a previously boiled cotton thread maintained under conditions identical to those used with nerve. No measurable amount of gas was evolved.

After incubation, that portion of the nerve which had been bathed in the fluid—approximately one-half by weight—was excised, thoroughly freed of adherent fat cells under a dissecting lens, homogenized in chloroform-methanol, and the total lipids were extracted, plated and counted as described previously (MAJNO and KARNOVSKY, 1958*a*). The amount of lipid obtained from one preparation averaged 2·5 mg.

RESULTS

The oxygen uptake of non-stimulated nerves was from 25 to 30 μl/100 mg fresh weight per hr.

Stimulation at 60 *pulses/sec.* The action potential showed little change over 2 hr, as exemplified in Fig. 2; the average drop in height was 7 per cent, and no change in threshold was observed. Oxygen uptake increased slightly during stimulation when both acetate and glucose were present in the medium. The effect of stimulation on respiration and on the specific radioactivity of the lipids is summarized in Table 1.

Stimulation at 300 *pulses/sec.* The action potential decayed far more rapidly than with 60 pulses; at the end of the first, second and third hours the average drop was 28 per cent, 73 per cent and 89 per cent respectively, and towards the end of the experimental period the threshold tended to increase. The effect on the respiration of the tissue and the specific activity of the lipids under these conditions is also summarized in Table 1.

DISCUSSION

Despite the large body of knowledge which has accumulated on the metabolic changes brought about in peripheral nerve by functional activity (MCILWAIN, 1955; HEALD, 1956; BRINK, BRONK, CARLSON and CONNELLY, 1952; BRINK, 1957; CRANEFIELD, BRINK and BRONK, 1957; MULLINS, 1953), changes in lipid biosynthesis have not, to our knowledge, been investigated. Only in the case of the central nervous system are a few data available (DAWSON and RICHTER, 1949; GEIGER, YAMASAKI and LYONS, 1956) which suggest that there is a relationship between functional activity and

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FIG. 1.—Two Warburg flasks modified for stimulation and recording of rat sciatic nerves. *Centre:* flask with electrode-bearing plug *in situ*. The socket for the stimulating plug is of larger taper than that which fits onto the manometer. The centre well is located under the latter socket, in order to make place for the electrodes. Dimensions: base 60 × 25 mm, height 25 mm, socket height 22 mm. *Right corner:* stimulating plug, made of lucite, bearing the two pairs of electrodes. At their base the electrodes are separated by a cross-shaped incision 10 mm deep, in order to prevent short-circuiting due to condensation on the lower end of the stopper.

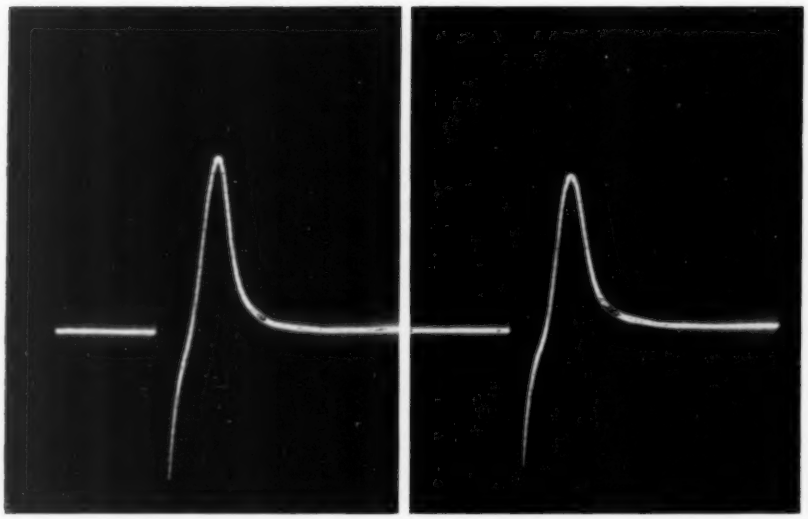

FIG. 2.—Compound action potential obtained from rat sciatic, stimulated at the rate of 60 pulses/sec, incubated at 37°C in Krebs–Ringer–phosphate medium containing glucose and equilibrated with 100% O_2. *Left:* at the start of the experiment. *Right:* after 2 hr. Further explanations in text.

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the metabolism of lipids. Thus DAWSON and RICHTER (1949) found that electric or emotional stimulation of mouse cerebral cortex caused a drop in the rate of phosphatide synthesis from [^{32}P] phosphate.

The preparation of peripheral nerve most commonly used in studies on the metabolic effects of stimulation has been the amphibian sciatic. In view of the paucity of data concerning the response of mammalian and particularly rat nerves to

TABLE 1.—CHANGES IN RESPIRATION AND INCORPORATION OF LABELLED BUILDING-BLOCKS INTO LIPIDS OF RAT SCIATIC NERVES DUE TO ELECTRICAL STIMULATION

Lipid precursor	No. of experiments	Specific activity of lipid extract* ± S.E.	*P*	Oxygen uptake*	*P*
	(A) *Stimulation at* 60 *pulses/sec*				
Acetate	7	119·6 ± 5·5	<0·01	123·8 ± 10·5	0·05
Glucose	6	112·4 ± 4·8	<0·05	100·9 ± 4·0	>0·8
Phosphate	4	98·3 ± 1·4	<0·5	†	
Choline	6	101·0 ± 4·1	<0·9	108·0 ± 7·9	>0·3
	(B) *Stimulation at* 300 *pulses/sec*				
Acetate	6	105·2 ± 8·9	<0·7	122·3 ± 8·9	<0·02
Choline	5	89·6 ± 3·6	<0·05	127·9 ± 10·7	>0·05

* Values for resting nerve set at 100. Respiratory values refer to the entire experimental period.
† Respiratory values omitted, since they were obtained in early experiments with less satisfactory flasks than those used subsequently.

electrical pulses, it was decided to provide the stimulating flasks with recording electrodes. This method also proved to be especially useful in that it permitted direct monitoring of the response to the applied stimulus.

With the arrangement adopted in our experiments, both ends of the nerve were suspended in the gaseous phase. Since diffusion of labelled substrate to these regions might have been poor, only the middle segment of the nerve was used for lipid extraction. Respiration, however, remained close to normal, since overall oxygen uptake was only 10–15 per cent lower than had been previously found (MAJNO and KARNOVSKY, 1958*a*) for nerves from rats of this age, using standard Warburg flasks in which the whole nerve was immersed. This agrees with findings of RODNIGHT and MCILWAIN (1954) who compared the oxygen uptake of tissue slices when either fully immersed or simply moist, i.e. suspended on a metal gauze.

The nerves were stimulated under two sets of conditions: one which could be considered relatively physiological, 60 pulses/sec for 2 hr, and one which led to definite fatigue of the nerve, i.e. 300 pulses/sec for 3 hr. 'Fatigue' refers here to progressive decrease in the area of the compound action potential.

At 60 pulses/sec a significant increase in respiration was found with only one combination of substrates, i.e. with glucose and labelled acetate (Table 1). However, if the respiratory data for all nerves stimulated at this frequency are combined (nineteen experiments) the rate of oxygen uptake by stimulated nerves is 111·6 ± 5·2 per cent that of resting nerves ($P < 0·05$).

At 300 pulses/sec the effect on respiration was more conspicuous, oxygen uptake

being 124·6 ± 6·6 per cent ($P < 0{\cdot}01$) for the eleven experiments using either [1-^{14}C] acetate or [1:2-^{14}C] choline with unlabelled glucose. In contrast with the rapid decay of the action potential, no respiratory 'fatigue' was apparent from the curve of oxygen uptake. A 15 per cent drop in the rate of respiration from the first to the third hour occurred with the stimulated as well as with the control nerves. These data are in general agreement with those of CRANEFIELD *et al.* (1957). The latter authors found that stimulation of rat sciatics at 500 pulses/sec at 35° caused a 12 per cent increase in the rate of oxygen uptake, and the increment associated with activity tended to reach a maximum in the neighbourhood of 200 pulses/sec, at which point it was 24 per cent above resting values. A closer comparison of our results with those of CRANEFIELD *et al.* cannot be made, because of the differences in experimental conditions. In particular, our experiments were carried out with a glucose-containing medium equilibrated with 100 per cent O_2, whereas those of CRANEFIELD *et al.* were performed with a medium equilibrated with an atmosphere containing CO_2.

The lipid extract obtained from our preparations can be considered true 'nerve lipid' free of any significant contribution from adipose tissue. As a refinement of the dissecting technique previously described (MAJNO and KARNOVSKY, 1958*a*) the nerve was cleaned, after incubation, under a dissecting lens, at an enlargement sufficient to resolve individual fat cells. Since endoneurial adipose tissue does not exist in the rat sciatic, the only possible source of extraneous lipid could be the epineurial membrane itself. We have found that the epineurium constitutes one-fifth by weight of the whole rat sciatic, and its lipid content is only about 1·5 per cent of its fresh weight.

Stimulation at 60 pulses/sec caused a significant increase in the biosynthesis of lipids from two of the precursors used: acetate and glucose (Table 1). A more definite effect was observed with acetate than with glucose, as might be expected from the fact that acetate carbon is more immediately available for lipogenesis than glucose carbon. In this connexion it is interesting to recall the work of MULLINS (1953), who studied the production of $^{14}CO_2$ by resting and electrically stimulated frog sciatics, in the presence of various [^{14}C]-labelled substrates. When either acetate or glucose were used, a *drop* in the rate of $^{14}CO_2$ production appeared during stimulation. This drop was 51 per cent in the case of acetate; 22 per cent in the case of glucose. Seven other substrates did not show this effect. If these results are considered together with those presented here, one might speculate that a proportion of both substrates, which at rest is oxidized to CO_2, is channelled into lipid biosynthesis during the conduction of impulses.

With labelled choline, stimulation at 60 pulses/sec caused no significant change in the rate of incorporation of the isotope (Table 1). The same was true when labelled phosphate was used. It should be noted here that ABOOD and GOLDMAN (1956) and ABOOD *et al.* (1958) studied the effect of stimulation on other aspects of phosphorus metabolism in frog nerves, and found significant quantitative changes in both organic and inorganic fractions.

In those experiments in which 300 pulses per second were applied, acetate and choline were incorporated to a lesser degree than with 60 pulses, as if a 'fatigue' also developed with respect to lipogenesis. It is of interest that whereas raising the frequency of stimulation from 60 to 300 pulses/sec emphasizes the increase in respiration, it diminishes lipogenesis from labelled precursors.

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In order to assign these experiments a significance with respect to the metabolism of nerves *in vivo*, it is necessary to make one assumption, i.e. that the changes observed as a consequence of stimulation *in vitro* are comparable to those of a nerve physiologically conducting *in vivo*. If this assumption is valid, the data suggest that the passage of impulses along the fibres accelerates the rate of lipid synthesis from two substrates, acetate and glucose. The total amount of lipid present need not change at all, of course, and in fact no measurable difference was found in the total lipid extracted from normal and stimulated preparations. Two other lipid building blocks are not significantly affected by the passage of impulses, at least not within the range of sensitivity of our techniques; both are moieties of phosphatide molecules: phosphate and choline.

This study should be further pursued by methods which would yield information on the cytological location, detailed chemical nature and metabolic function of the lipids affected by stimulation. An approach of this nature might provide clues to problems of demyelination.

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SUMMARY

Modified Warburg flasks are described, with which it is possible to apply electrical pulses to peripheral nerves and to record the resulting action potential, in the course of incubation.

Using these flasks, rat sciatic preparations were incubated and stimulated in the presence of each of four labelled lipid building blocks (acetate, glucose, phosphate, choline). The total lipids of the nerves were subsequently extracted, their radioactivity measured, and compared with that of lipids from non-stimulated preparations.

In general, stimulation increased the oxygen uptake. When the nerves were stimulated within a relatively physiological range of frequency (60 pulses/sec for 2 hr) there was a significant increase in lipogenesis from acetate and from glucose. When the nerves were submitted to stimulation leading to 'fatigue' (300 pulses/sec) lipogenesis from acetate remained unchanged, and the incorporation of choline was significantly decreased. The significance of these findings is discussed.

Acknowledgements—The assistance of Miss DONA NELSON is gratefully acknowledged.

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COMPARATIVE HISTOCHEMISTRY OF LIPIDS IN RELATION TO MYELINATION IN RABBIT BRAIN

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PAST investigations into the process of myelination in the central nervous system have been either wholly biochemical or restricted to special circumscribed areas. The work of BRANTE (1949) and of EDGAR (1955, 1956), for instance, was almost entirely biochemical. The latter investigator, however, made some histological observations with the traditional Weigert-Pal method as an indicator for the development of myelin in rabbit brain. This is a suitable material for histochemical studies, not only because myelination takes place entirely after birth, but also on account of EDGAR's findings, especially with regard to the late appearance of cerebrosides in relation to sphingomyelin.

In the studies reported in this paper, bearing in mind the chemical composition of the myelin sheaths, we used relatively few of the available histochemical techniques for lipids.

EXPERIMENTAL

Material and methods

Rabbits of 1, 5, 7, 11, 15, 21 and 28 days, and 2 and 5 months of age, as well as adults (older than 8 months), were used. No special care in breeding was taken. The animals were killed with chloroform and the brain immediately removed. The skull was opened from the occiput, care being taken to preserve the olfactory bulb and the paraflocculi. Coronal sections were made, through the whole extent of the brain, and alternate blocks were fixed according to the requirements of the individual techniques. As an alternative, the twin halves of a single block were used for two different methods. It was found easier to section the brain into blocks if it was first frozen for a short while at −20°C. The methods used were:

Baker's acid haematein (*BAH*). This was used according to the original directions (BAKER, 1946). Two points, already noted by BAKER, were of great importance in obtaining good results. First, gelatin-embedded blocks permitted better sectioning and, secondly, the results were much better with 8–10 μ sections. With this technique phospholipids and certain proteins such as legumin, caseinogen and mucin stain positively.

Pyridine extraction (*PE*). This was used as a control for the previous method. Substances positive with BAH, other than phospholipids, resist extraction and continue to stain, often more strongly than before. The combined PE and BAH method was controlled by the use of Sudan black B after extraction.

Copper phthalocyanin (*CuPC*). (KLÜVER and BARRERA, 1953; PEARSE, 1955.) Frozen sections, after fixation in 15% (w/v) formalin containing 1% $CaCl_2$, were stained for 16 hr in 0·1% Luxol fast blue (Du Pont) in absolute ethanol at 56°. After washing in alcohol and water, the sections were differentiated in aqueous 0·05% lithium carbonate for 90 min. Longer differentiation made no

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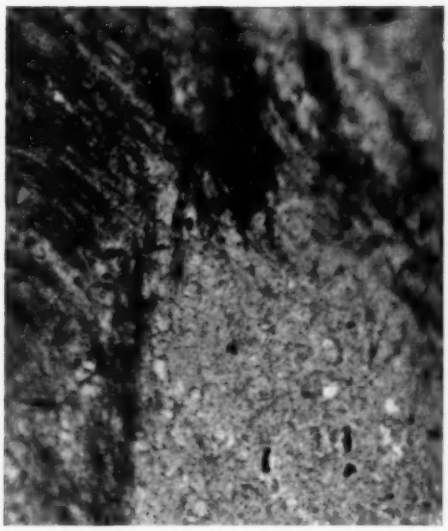

FIG. 1.—One day old rabbit, showing stained myelinated fibres in the medulla oblongata. BAH. ×300.

FIG. 2.—Eleven day old rabbit. Myelinated fibres are faintly stained in the telencephalon (corpus callosum). At upper right the intracellular reaction is shown. BAH. ×150.

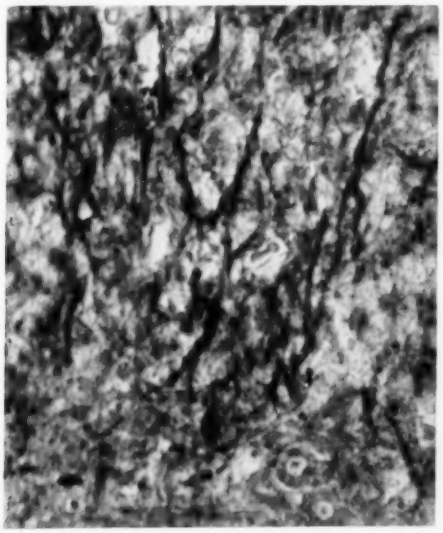

FIG. 3.—Five months old rabbit. Granular and Purkinje cells, layers of the cerebellum. The aspect observed is similar to the adult one. Besides the myelinated fibres reaction there is also some ill-localized staining in the granular layer. Purkinje cells moderately stained. BAH. ×300.

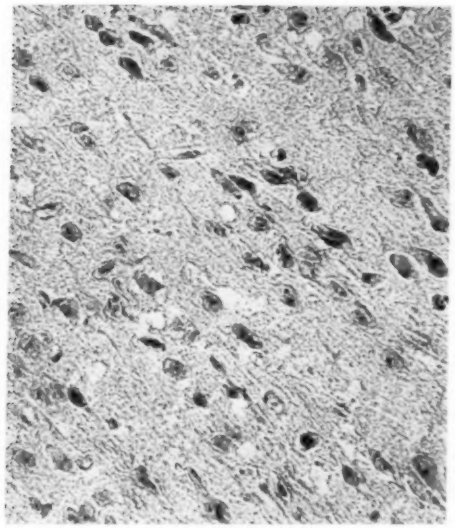

FIG. 4.—Seven day old rabbit; cerebral cortex. The section is negative, coloured pale yellow. The cells appear stained on account of refraction. PE. ×300.

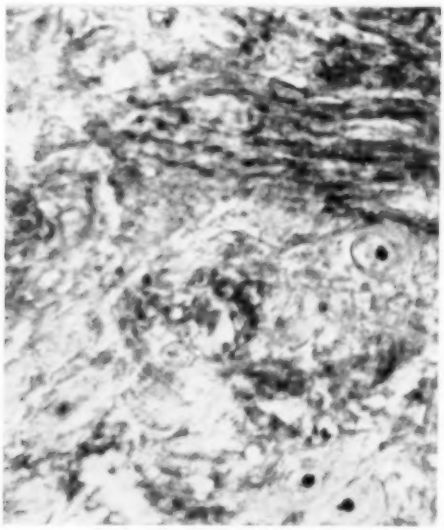

FIG. 5.—Five months old rabbit. Medulla oblongata, showing the strong nucleolar reaction and the colourless cytoplasm. Strong residual staining of the myelin sheaths. PE. ×600.

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FIG. 6.—Seven day old rabbit. Telencephalon, showing corpus callosum and cornu Ammonis. No reaction is observed (carmalum counterstaining). CuPC. ×100.

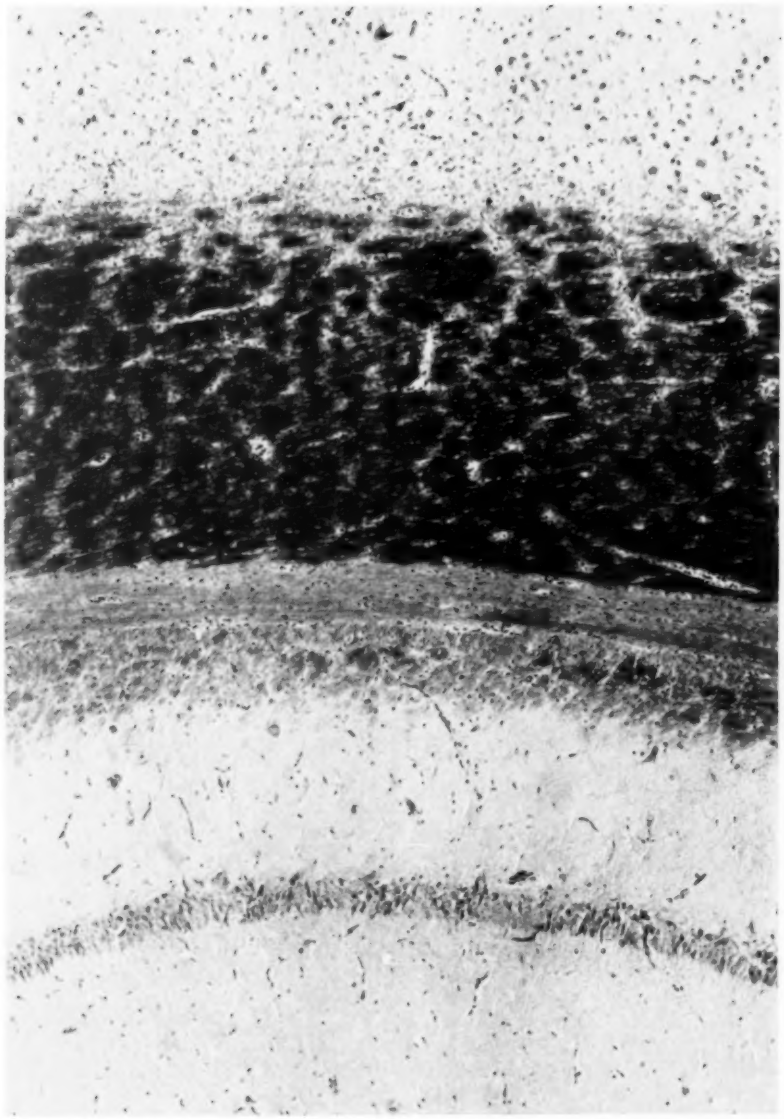

FIG. 7.—Five months old rabbit. Area corresponding to the one shown in the previous figure. Aspect similar to the adult. CuPC. ×100.

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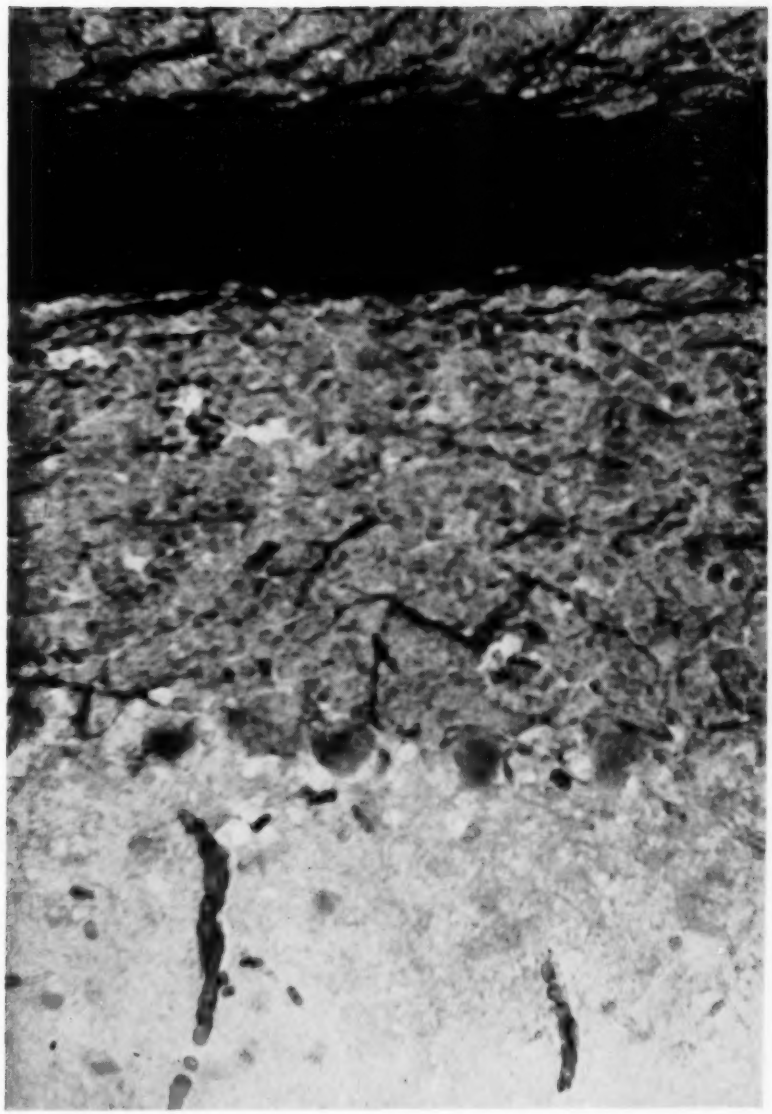

FIG. 8.—Five months old rabbit. Cerebellum, showing intense reaction of the white matter and faint reaction of the molecular layer. Purkinje cells also stained. Irregular staining in the granular layer similar to Fig. 3. CuPC. ×400.

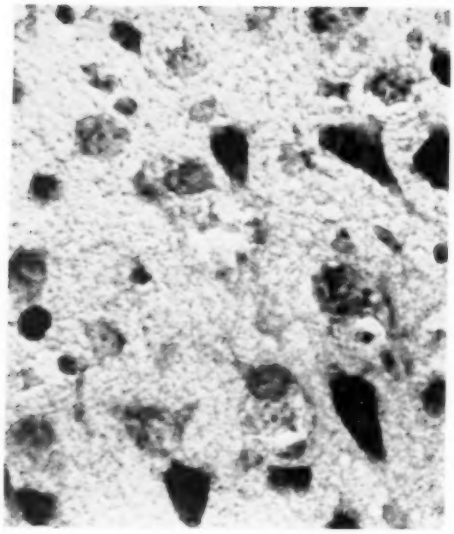

FIG. 9.—Fourteen day old rabbit; telencephalon. Shows cortical neurons and diffuse (metachromatic) staining of the background. Feyrter's method. ×400.

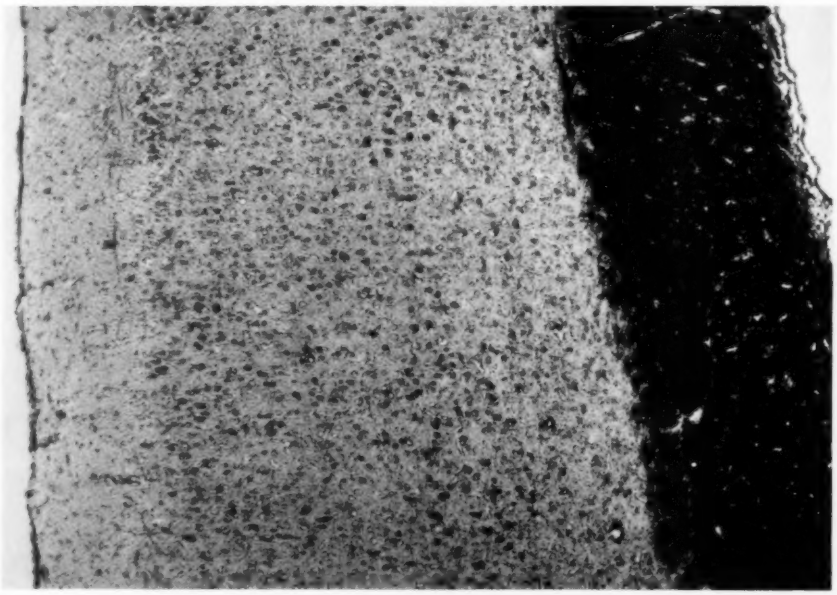

FIG. 10.—Five months old rabbit; telencephalon. Shows strong (metachromatic) reaction of corpus callosum and of the fine cortical fibres. The background is only faintly stained. Feyrter's method. ×75.

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FIG. 11.—Five months old rabbit; cerebellum. There is a sharp distinction between white matter (blue-violet) and the other layers (reddish-pink). No cellular staining. Okamoto. ×100.

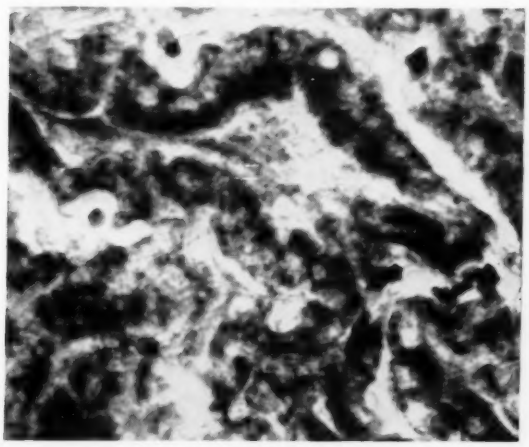

FIG. 12.—Five months old rabbit; choroid plexus, lateral ventricle. Intense intracellular reaction, nuclei unstained. BAH. ×600.

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difference to the final result. Sections were subsequently dehydrated in alcohol, cleared in xylene and mounted in polymerized styrene resin (DPX), with and without previous counterstaining with carmalum. Phospholipids stain blue by this method.

Feyrter's mounting-staining method. (FEYRTER, 1936.) Formalin-fixed frozen sections were fixed to slides, dried by blotting and covered with 1% thionin in 0·5% aqueous tartaric acid for 5 min. A coverslip was then applied and ringed with asphalt varnish, after carefully drying the edges. This characteristic metachromatic reaction takes some time to develop and the process can be accelerated by heating at 60°. Cerebrosides and phosphatides stain metachromatically (red).

Okamoto's mercury-diphenylcarbazone method. This was cited by UEDA (1952) as the 'common' test. Formalin-fixed frozen sections were treated with acetone containing 2% magnesium chloride for 48 hr at room temperature. After washing they were put in the following solution for 24 hr in the refrigerator: 10 ml of a saturated solution of mercuric nitrate in 60% (v/v) alcohol containing 0·2 ml of 0·2% sodium chloride. A thorough washing of the sections followed and they were then treated with a 5% aqueous solution of potassium iodide, for 4–5 min. A fresh washing preceded the use of a 2% sodium acetate solution, for 10 min, immediately followed by diphenylcarbazone (saturated solution in 80–100% (v/v) alcohol), for 10 min. The sections were then washed and mounted with glycerine jelly. The authors state that not only are phospholipids positive, but also cerebrosides and 'some other material besides lipids'. The final colour is bluish-violet.

The distinction between phospholipids and cerebrosides is made by means of a cold pyridine extraction, for 48 hr, during which the cerebrosides are supposed to be removed.

Further differentiation between the main classes of phospholipids involves the following treatments:

(a) For demonstration of sphingomyelin alone: formalin-fixed frozen sections are rinsed with acetone and extracted at room temperature with ether for 48 hr, followed by a new rinse in acetone and processing as for the 'common' method, from the mercuric nitrate stage.

(b) For demonstration of both sphingomyelin and lecithin: after treating the sections until the mercuric nitrate stage, as in the 'common' method, they are extracted with ether at room temperature for 48 hr, and the sequence is then followed as described.

Elftman's controlled chromation. (ELFTMAN, 1954.) Tissue blocks were submitted to 2·5% potassium dichromate in 0·2 M-acetate buffer, pH 3·5, for 18 hr at 60°, followed by alcoholic dehydration and paraffin embedding. As the tissue becomes brittle after this procedure better results can be achieved by using sections about 8 μ thick. Serial sections were stained alternatively in Sudan black B in propylene glycol, according to CHIFFELLE and PUTT (1951), and by 0·1% haematoxylin in 0·2 M-acetate buffer, pH 3·0, containing 0·005% potassium ferricyanide, for 2 hr at 56°. Chromated lipids are stained by both Sudan black B and by haematoxylin, non-chromated lipids only by Sudan black B, and non-lipid chromated substances only by haematoxylin, as stated by the author. It was found necessary in the course of our work to oxidize the haematoxylin to haematein. Mercuric oxide was employed for this purpose, in order to avoid inhibition of staining which occurs when iodate is used.

Periodic acid–Schiff reaction. Formalin-fixed frozen sections were treated for 5 min with 0·5% periodic acid, washed in distilled water, developed in Schiff's reagent for 10 min, and washed for 10 min in running water. Mayer's haemalum was used as a counterstain, followed by alcoholic dehydration and mounting in DPX. Controls for pre-existing aldehydes were provided by blocking with hydroxylamine (10 g hydroxylamine hydrochloride, 20 g sodium acetate, 40 ml water) (PEARSE, 1953), at room temperature for 3 hr. A positive reaction is due to the presence of 1:2-glycol, 1-hydroxy-2-amine, 1-hydroxy-2-alkylamine or 1-hydroxy-2-ketone groups (cf. LISON, 1953). Glyco- and phospholipids are also known to be positive (PEARSE, 1953; WOLMAN, 1950).

Alkaline tetrazolium reaction. (PEARSE, 1954.) Formalin-fixed blocks were embedded in paraffin in the usual way. Sections (5 μ) were rehydrated, and incubated for 90 min in a solution of Blue tetrazolium (BT) in 0·1 M-NaOH-glycine buffer at about pH 12 and at 60°. Nuclei were sometimes counterstained with Mayer's haemalum. After processing, the slides were mounted in glycerin-jelly.

RESULTS

It is not our intention to give a detailed anatomical description of the numerous tracts of the brain, but to deal instead with each of the main divisions (telencephalon, mesencephalon and metencephalon) as a whole, occasional mention being made when

necessary of particular nerve tracts or cells. This procedure simplifies comparison between the histochemical results and the available biochemical information.

The results are presented under the headings of the separate methods.

Baker's acid haematein

In the 1 day-old rabbit brain, a diffuse reaction is observed throughout the neuropil in all parts of the central nervous system in the form of a very fine, faintly granular staining. Only in the medulla oblongata can a few fine fibres be detected at the *formatio reticularis* and the tractus *spino-cerebellaris dorsalis* (Fig. 1). In the cerebellum there is a faint reaction in the molecular layer, in the region of the Purkinje cells. There is, however, already an outlining of the areas in which the myelin is about to develop, as these are somewhat more intensely stained than the surrounding neuropil.

After 5 days from birth the positive fibres detected by this method can be found in all parts of the metencephalon. In the white matter of the cerebellum the reaction is still indefinite by comparison with the pons and medulla oblongata where the fibres are already well characterized and distinct from the surrounding structures. The telencephalon and the mesencephalon are negative, except for the diffuse reaction already described which is more intense in the areas subsequently occupied by myelinated fibres. The fibrosa and glomerulosa layers in the olfactory bulb are coloured dark-grey. Not until the seventh day of life do these areas begin to show a definitely positive reaction in their main fibre tracts as shown by the corpus callosum, corona radiata, capsula interna and anterior commissure. Olfactory fibres appear at this time in the olfactory bulb. At the same time the myelin is already strongly stained in the metencephalon.

From the eleventh day onwards a strong reaction is found (Fig. 2), which gradually becomes localized in the finest fibre tracts. The process continues until the period between 2 and 5 months of life when it seems to be histochemically complete.

By the twenty-first day of life a positive reaction occurs in the granular layer of the cerebellum (Fig. 3). As this area is filled with cells, there is always the possibility that the reaction is intracellular, though this is difficult to establish even under high-power magnifications.

The completion of myelination is shown histochemically by the appearance of the finest tracts in the cerebral cortex, the last to become positive.

Pyridine extraction

From the first to the seventh day of life the PE control for the BAH method prevents staining of the lipid tissue components responsible for the positive reaction. There is, at the same time, generalized nuclear and nucleolar staining (Fig. 4). By the eleventh day the metencephalon begins to show residual staining of the myelin sheaths after PE and this occurs in the mesencephalon and telencephalon a little later, around the fifteenth day after birth. This residual staining is especially marked in the main tracts and it increases in parallel with the BAH staining, with similar progression from the metencephalon towards the telencephalon. It is stronger in the older animals and reaches its maximum between 2 and 5 months (Fig. 5).

The residual staining after PE was shown to be positive with the Sudan black B and CuPC techniques (q.v.).

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Copper phthalocyanin

In the 1 day-old rabbit only the few fibres shown by the BAH method in the medulla oblongata were stained. At 5 days the reaction extended to the pons, although the cerebellum was still negative. At 7 days the three components of the metencephalon gave positive reactions and myelination was observed to begin in the telencephalon and mesencephalon. At first, staining was observed only in the fibres surrounding the lateral ventricle while the more anterior parts (Fig. 6) were completely negative. The olfactory bulb showed no staining until the eleventh day when the glomerulosa and medullaris layers stained faintly. From then on the reaction became more and more intense, reaching the adult level at between 2 and 5 months (Fig. 7). The findings in the granular and molecular layers of the cerebellum (Fig. 8) are equivalent to those demonstrated by the BAH method.

Feyrter method

From the age of 24 hr until about 28 days there is a noticeable metachromatic staining of the background (Fig. 9) which becomes progressively weaker. Even so the future myelinated areas, in the younger animals, are stained in a stronger shade of pink than the remainder of the background. Definite fibres appear by the fifth day in the metencephalon and a little later, by the seventh day of life, in the telencephalon and mesencephalon. As the fibres become more strongly metachromatic (Fig. 10) the background reaction becomes less intense.

The general pattern of evolution of the myelinated fibres is similar to the one already described in the previous methods.

Okamoto's method

The 1 day rabbit brain already shows staining of the regions where the myelinated fibres are to be found. The shade is not violet-blue, however, but reddish-pink against a pink background. This finding applies to all parts of the brain. The characteristic violet-blue colour of the diphenylcarbazone-$Hg^{2(+)}$ complex occurs first in the metencephalon at the seventh day and by the eleventh day it is found in the anterior parts. With increasing age the colour grows deeper, but the background staining increases simultaneously. This makes distinction between positive and negative areas very difficult, especially when there is no sharp definition between the grey and white matter, as for instance in the radiation of the corpus callosum. The finest fibres are then lost against the intense red background. In some cases the distinction is made more easily, as in the cerebellar circumvolutions (Fig. 11) where the differentiation between grey and white matter is clearly observed.

Elftman's controlled chromation

According to the original author's views, only structures positive with Sudan black B and haematoxylin can be considered to be phospholipids. The account which follows refers to components reacting positively with both methods. There is always a finely granular staining of the background with Sudan black B while haematoxylin gives a diffuse reaction. From the first to the eleventh day of life intracellular staining is irregular; after that time it is consistently observed in the mitral cells of the olfactory bulb, the neurons of the metencephalic nuclei, the Purkinje cells of the cerebellum and in the cortical neurons of the telencephalon. It is shown either as an irregular and

more or less coarse granular deposition of Sudan black B, or as a diffuse staining of the cytoplasm. The pattern exhibited by fibre tracts follows the general description related above, with the earliest demonstration of metencephalic myelin at around the seventh day. The choroid plexus, however, is positive at an earlier stage (Fig. 12) and its reaction progressively increases until the adult level is reached.

Periodic acid–Schiff and alkaline tetrazolium reactions

These two methods will be considered together on account of the parallelism of their results. Staining of the myelinated fibres with these methods is similar to the general pattern described. When the myelin fibres are definitely shown by means of the lipid-staining techniques the PAS and alkaline tetrazolium reactions become positive in the same regions. These two reactions become progressively more intense as the age of the brain increases, and at 2–5 months, as described by HESS (1953), the fibres stain brilliantly red by the PAS method. This author (1954) and LEBLOND (1950) have referred to the PAS-positive background staining in the brain. HESS attributed this to a neutral mucopolysaccharide constituent. This view is shared by WOLMAN (1956) and our results, though they are in general agreement with his, should be extended by more specific work. This relationship was not found with respect to the choroid plexus and the lipid-containing cells gave positive PAS and alkaline tetrazolium reactions from the first day after birth. These increased with age, becoming gradually stronger and reaching a maximum at 5 months.

DISCUSSION

Our results with the BAH and CuPC methods show stainable fibre tracts at an earlier stage than shown by EDGAR (1955, 1956) in the same material. This is due solely to the greater sensitivity of these two techniques when compared with the Weigert–Pal method. We were able to demonstrate myelin fibres by the BAH and CuPC methods as early as 24 hr after birth, in the medulla oblongata. Later on, however, the BAH method showed myelin tracts before the CuPC method, as observed in the 5 and 7 day old rabbits. This apparent anomaly is probably due to the advanced stage of myelination in the newborn medulla since everywhere else the CuPC method shows developing myelin at a later stage than the BAH method.

It is known, from biochemical studies, that practically only sphingomyelin is present in the white matter of the young rabbit brain, cerebrosides being present in too small a concentration to influence the reaction. They increase notably after the ninth day of post-natal existence. These observations account for the difference between the two methods since the standard (alcoholic) CuPC method does not stain sphingomyelin.

We were able to demonstrate clearly the earlier development of myelin in the phylogenetically older parts of the brain, suggested by the biochemical observation of higher concentrations of cerebroside and sphingomyelin in these areas. From the histochemical point of view myelination was completed in the period between 2 and 5 months of life (cf. EDGAR, 1955, 1956; FOLCH, 1954).

The diffuse staining of the neuropil, most easily recognized by means of the Feyrter reaction, probably indicates a diffuse localization of sphingolipids prior to their incorporation into the myelin fibres. This suggestion is supported by the fading of neuropil staining at the same time as the reactions of the myelin sheaths became positive.

A point of interest is related to our findings with the pyridine extraction method. This method, combined with BAH, gives a negative result where staining is due to phospholipids. Up to the eleventh day the BAH reaction of the various fibre tracts is reversed by PE. After this there is an ever-increasing amount of residual BAH staining of the myelin sheaths, strongest in the main tracts. The abrupt occurrence of residual staining, at the time when rapid deposition of sphingomyelin and cerebrosides begins, needs further explanation. It is possible that the PE method is less effective when a higher proportion of lipid is present in the myelin sheaths, but it is also possible that the BAH method stains cerebrosides in the tissues even if it does not do so *in vitro*. Since the CuPC and Sudan black B methods are both positive in the myelin sheaths after PE residual staining, cerebroside is necessarily present.

The presence of diffuse intracellular phospholipid, previously described by GLEES (1955), requires some comment. This was apparent in all parts of the brain from the fifth to the seventh day of life, particularly in the mitral cells of the olfactory bulb and in the neurons and glial cells of other regions. Neuronal staining was, as a rule, more intense than glial staining. In our series of brains the intensity of intracellular staining reached its maximum at the eleventh day (Fig. 2) and thereafter decreased steadily. At 21 to 28 days after birth the adult pattern appeared, that is to say the mitral cells became negative and in most of the other cells only a faint trace of cytoplasmic staining remained. Exceptions were the metencephalic neurons, the Purkinje cells of the cerebellum and the cells of the molecular and granular layers (Fig. 3). In this last case it was difficult to establish the precise localization of the BAH-positive material. Intracellular staining was observed to be patchy, seldom affecting all the components in a single section, and usually most intense in cells near myelinated regions. It was always reversed by pyridine extraction, whether demonstracted by the BAH method or by Feyrter's technique. With the Okamoto method the intracellular staining was never observed. Its precise nature and function remain obscure, but we feel that it is in some way connected with myelination.

In the case of the granular and molecular layers of the cerebellum the results of the BAH and CuPC methods suggest that two different lipids are responsible. Working with monkeys, ROBINS, EYDT and SMITH (1956) showed that the sphingomyelin content of these layers is much lower than in the subadjacent white matter. These authors suggested that gangliosides might be the main components of the granular layer and these are well stained by the CuPC method (PEARSE, 1957), although the mechanism by which they stain is not understood.

CONCLUSION

The changes we observed by histochemical methods in the lipids of the myelin sheaths of the developing rabbit brain were in close agreement with the biochemical findings of EDGAR (1955). Still better agreement might be achieved with the development of more sensitive and specific histochemical methods for the various lipid fractions.

SUMMARY

(1) Histochemical techniques for phospho- and glycolipids were applied to the problem of myelination in the developing rabbit brain.

(2) Myelinated fibres were first demonstrable, as early as 24 hr after birth, in the

medulla oblongata. The process of myelination was histochemically completed between the second and fifth months.

(3) The earlier development of myelin in the phylogenetically older parts of the brain was clearly shown.

(4) Diffuse staining of the neuropil occurred in brains from 24 hr to 28 days old. This was considered to indicate temporary storage of sphingolipid prior to its incorporation in the myelin sheaths.

(5) Diffuse intracellular phospholipid was especially apparent in all parts of the brain from the fifth to the seventh day. The adult pattern emerged at the twenty-eighth day. The nature and function of this lipid remain obscure.

(6) The histochemical results, in general, agreed well with the findings of reported biochemical studies on similar material. In particular, the striking increase in cerebroside content of the myelin sheaths, observed biochemically at and after the ninth day, was reflected in the histochemical tests.

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DISPOSITION OF γ-AMINOBUTYRIC ACID ADMINISTERED TO MAMMALS*

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MAMMALIAN brain contains considerable amounts of γ-aminobutyric acid (GABA) (AWAPARA, LANDUA, FUERST and SEALE, 1950; ROBERTS and FRANKEL, 1950; UDENFRIEND, 1950). Traces of GABA have been reported to be present in blood and urine (DENT, 1948; CRUMPLER, DENT and LINDAN, 1950) gastric juice (GILLIGAN, MOOR and WARREN, 1951) and saliva (MOOR and GILLIGAN, 1951). Very little GABA has been found in tissues other than brain (TALLAN, MOORE and STEIN, 1954). GABA has been found by BAZEMORE, ELLIOTT and FLOREY (1956, 1957) to be the main component of extracts from brain which shows 'Factor I' activity, that is, inhibition of the activity of the stretch-receptor neurone of the crayfish. Marked changes in the electrical responses of cat cerebral cortex on topical application of GABA (PURPURA, GIRADO and GRUNDFEST, 1957; IWAMA and JASPER, 1957) or after intracartoid injection (MARRAZZI, HART and RODRIGUEZ, 1958), seem to indicate that this substance may play an important role in brain physiology.

In the course of experiments on the injection of GABA into human subjects and animals it was noted that only brief effects were produced. The present work was carried out in order to discover, by determinations of Factor I activity, the distribution and fate of injected GABA.

EXPERIMENTAL

Animals and dosage. Rats, weighing from 175 to 210 g, were fasted overnight and received 500 mg/kg body weight GABA, as a 50% solution in water, intraperitoneally. In a few experiments GABA was given orally at the same dose level; the solution was mixed with 0·2 ml of strong sucrose solution, and the rats drank it, without loss, from a syringe. At various intervals after administration the animals were decapacitated and the brain (cerebral hemispheres) kidney, liver and skeletal muscle (leg) were removed as soon as possible after death and rinsed with saline. Urine was taken directly from the bladder immediately after death. When it was desired to measure the total urinary excretion, the animals were kept on a ¼ in. chicken-wire platform in a beaker. The animal was anaesthetized with ether before removing it from the beaker. The urine was washed from the beaker and platform, with buffered crayfish saline, and urine collected from the bladder was added to this solution.

A cat under chloralose anaesthesia (70 mg/kg body weight) received an intravenous injection of GABA, 200 mg/kg body weight. Blood samples, about 4 ml each, were taken from the carotid artery at various times. A needle was inserted into the cisterna magna, before the GABA injection, to obtain samples of cerebrospinal fluid.

An unanaesthetized rabbit received a similar dose of GABA intravenously. Blood samples were collected by tapping the ear veins.

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Preparation of tissue for assay. Samples of the organs were homogenized in nine times their weight of potassium-free unbuffered crayfish saline containing 5mM-glutamate (ELLIOTT and VAN GELDER, 1958). The suspensions were then placed in a boiling-water bath for 15 min.

The clotted blood was centrifuged and the electrolyte concentration of the serum was adjusted to that of complete buffered crayfish saline by adding one-tenth of its volume of an adjustment solution and of 0·11 M-tris-maleate buffer (ELLIOTT and VAN GELDER, 1958). The pH was adjusted approximately to 6·5 with N-HCl. Buffered crayfish saline, containing 5 mM-glutamate, was added until the final volume was three times that of the original serum. The solution was placed in a boiling-water bath for 15 min and the congealed mass was homogenized.

Urine samples, collected from the bladder, were diluted with buffered crayfish saline. All samples were stored at −20°C until they were assayed.

Assay. Factor I activity (inhibition of the spontaneous responses of the crayfish stretch receptor neurone) was assayed by the method of ELLIOTT and FLOREY (1956) as modified by ELLIOTT and VAN GELDER (1958). The Factor I activity is expressed in terms of the amounts of GABA which would show the same activity.

RESULTS

In Fig. 1 the concentration of GABA in the serum of rats, cat and rabbit are plotted on a log scale against the time following administration . The results indicate that absorption from the peritoneum is rapid but the GABA leaves the blood again very rapidly, its concentration being approximately halved every 20 min. The concentration in the serum from rats at various intervals after oral feeding of GABA does not rise as high as after intraperitoneal injection and remains rather constant for a considerable time.

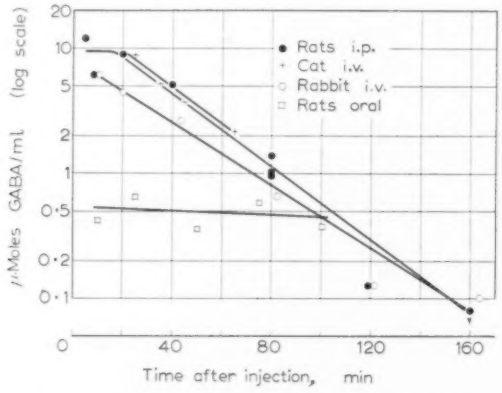

FIG. 1.—Concentration of Factor I (as GABA) in serum after administration of GABA. Rats received 500 mg GABA per kg body weight; intraperitoneally or orally. The cat and the rabbit received 200 mg/kg intravenously. ↓ No activity detectable. No Factor I was detectable in blood before GABA administration.

The concentrations of GABA which appeared in other tissues of the rat are shown in Fig. 2. High concentrations are found in kidney, liver and muscle and very high concentrations in the urine shortly after the injection. These concentrations thereafter fall. Kidney shows the highest early concentration and the most rapid fall. There is some delay before the activity in the liver begins to fall rapidly. The concentration in muscle does not reach as high a level as in the other tissues and falls more slowly.

In brains of uninjected rats the Factor I activity is equivalent to that of 3·3 ± 0·4 (S.D.) μmoles/g. No significant elevation above this level could be detected after

injection of GABA even when the activity in the blood was much higher than that in the brain.

In the cerebrospinal fluid of one anaesthetized (chloralose) cat, activity equivalent to 0·2 μmoles/ml was found at a time, 30 min after injection of GABA, when the

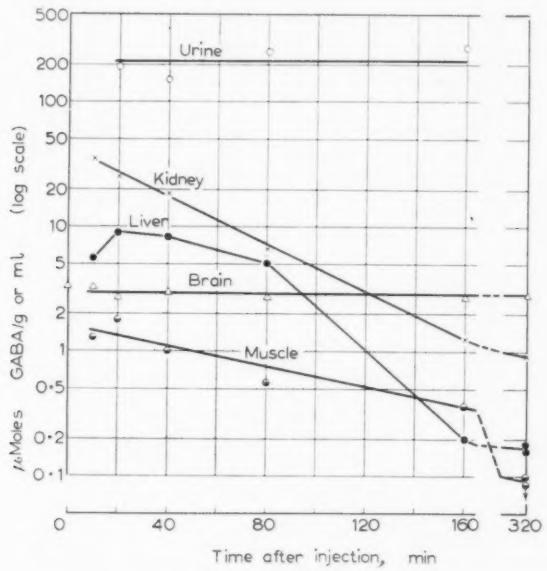

FIG. 2.—Factor I concentration (as GABA) in rat urine, kidney, liver, muscle and brain after intraperitoneal injection of GABA, 500 mg/kg body weight. ↓ No activity detectable. No activity was detectable in urine or tissue other than brain in animals which did not receive GABA. Known amounts of GABA, 0·3 or 1·5 μmoles per g tissue, added to homogenates of liver, muscle or kidney were well recovered. In urine and kidney, however, concentrations of less than about 0·3 μmole/ml or g could not be reliably estimated because urine and kidney extracts, when repeatedly applied at low dilutions, interfere with the normal behaviour of the stretch receptor. It is possible that very small amounts of GABA may be present in normal kidney and urine (cf. DENT, 1948).

blood serum contained about 7 μmoles/ml. In another cat, which had received two large injections and had been subjected to various procedures, 0·1 μmoles/ml was found in the cerebrospinal fluid when the blood serum contained 7·5 μmoles/ml. In unpublished experiments with H. JASPER, no activity was detectable (less than 0·01 μmoles/ml) in the cerebrospinal fluid of an unanaesthetized monkey which had received 200 mg/kg of GABA 30 min previously. The blood of this animal, taken about 15 min later, contained about 2·6 μmoles/ml.

In experiments in which it was attempted to collect all the urine excreted, activity equivalent to 26–60% (average 45%) of the total Factor I activity of the injected GABA was present in the urine 320 min after intraperitoneal injection of 500 mg/kg.

DISCUSSION

The data seem to indicate that while GABA is easily absorbed from the peritoneum or stomach, rapidly distributes itself in the blood and tissues and is rapidly excreted in the urine, the blood-brain barrier effectively prevents any appreciable increase in the

GABA content of the brain of the adult animal. The possibility that GABA entering the brain is rapidly destroyed seems unlikely since, though mechanisms are known whereby GABA can be broken down, (BESSMAN, ROSSEN and LAYNE, 1953; ROBERTS and BREGOFF, 1953; ROBERTS and BAXTER, 1958; TSUKADA, NAGATA and TAKAGAKI, 1957; SUGIURA, 1957) *in vitro* experiments (ELLIOTT and VAN GELDER, 1958), have shown that brain suspensions do not inactivate GABA at an appreciable rate and brain slices absorb large amounts of exogenous GABA without destroying its activity. The appearance of very small amounts of GABA in the cerebrospinal fluid of the cat indicated slight penetration from the blood but the blood-csf barrier might have been modified by the anaesthetic. Following intracarotid injections into lightly anaesthetized cats, MARRAZZI *et. al.* (1958) have observed inhibitory effects on cortical synapses which seems to indicate that some GABA must pass from the blood into the brain under their conditions. It might be mentioned that we have not been able to detect Factor I activity in cerebrospinal fluid samples from patients suffering from a wide variety of conditions. (The minimum concentration detectable was 0·05 μmoles/ml.)

The appearance of GABA in liver and muscle after injection is not considered to be due to an active absorption process. No such absorption was found by ELLIOTT and VAN GELDER (1958) with kidney and liver slices or diaphragm though diffusion from the medium into the tissues occurred. The concentration in the liver or muscle never becomes higher than the maximum level reached in the circulation. The delay in the disappearance of GABA from liver may be attributable to characteristics of the hepatic circulation; it would take time for the GABA-containing blood in the liver to be completely replaced. The concentrations in muscle never reach such high values as in the blood, liver and kidney, and fall more slowly. Presumably diffusion in the tissue spaces is slower than the rate of disappearance from the blood. The very high concentration found in kidney shortly after injection probably reflects its activity in concentrating GABA in the urine. The incomplete recoveries in the urine may indicate that excretion occurs with appreciable conversion of GABA into an inactive or less active substance. PISANO, MITOMA and UDENFRIEND (1957) have shown that a transamidination reaction mechanism, involving GABA and an amidine donor, exists in the kidney. The substance formed, γ-guanidinobutyric acid, was reported to have only one thirty-fifth of the activity of GABA when applied to the stretch receptor. The high rate of excretion combined with a rate of absorption from the stomach, which is slower than that from the peritoneum, would explain the facts that oral administration of GABA results in a smaller rise and a slower fall in blood concentration than is found after intraperitoneal administration.

The results obtained in this study are comparable with those obtained by CHRISTENSEN, ASPEN and RICE (1956) and NOALL, RIGGS, WALKER and CHRISTENSEN (1957), who injected α-amino*iso*butyric acid and other non-metabolizable α-methyl amino acids into rats. Following intraperitoneal administration into rats, high concentrations of these amino acids could be detected in blood, liver or skeletal muscle; the concentrations in muscle were less than those found in either blood or liver. The fall in the concentrations of the methyl amino acids was slower than we found with GABA. LAJTHA (1958) has recently shown that following injection of lysine into adult mice, the lysine content of liver reaches levels far higher than can be accounted for by simple equilibration with the plasma; less appears in muscle and very little in brain.

We conclude from these results that effects of GABA administered intravenously

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or intraperitoneally must be expected to be shortlived and virtually no effects on the brain are to be expected. This conclusion is in accord with the observations of PURPURA, GIRADO, SMITH and GOMEZ (1958), that under ordinary circumstances systemically administered GABA does not produce electrophysiological effects in the brain but does so in local regions where the blood-brain barrier has been destroyed.

SUMMARY

Following intraperitoneal injection of γ-aminobutyric acid (GABA) into rats, this substance when determined by assays of Factor I activity, rapidly appeared in the blood, kidney, liver and muscle. The GABA content in the blood and kidney fell again rapidly, the concentrations being halved every 20 min; the activity in the liver was maintained for about 80 min and then fell rapidly. The concentration reached in muscle was lower than in the other tissues and fell more slowly. No changes in the Factor I activity of the brain occurred. Oral administration of GABA resulted in a considerably smaller increase in blood concentration and a slower fall.

The GABA content of the urine became extremely high shortly after administration and after 320 min a value for the activity corresponding to 26–60 per cent of the dose administered was found in the urine.

Similar changes in blood content were obtained after intravenous administration of GABA to an anaesthetized cat and an unanaesthetized rabbit. Traces of Factor I activity were found in cerebrospinal fluid from anaesthetized cats, none in fluid from an unanaesthetized monkey although the activity in the blood was very high.

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INDIVIDUAL, STRAIN AND AGE DIFFERENCES IN CHOLINESTERASE ACTIVITY OF THE RAT BRAIN*

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WE have previously published data (ROSENZWEIG, KRECH and BENNETT, 1958) which indicate the existence of a relationship between the level of cholinesterase (ChE) activity in rat cortex and adaptive behaviour. Since our interest was in the normal enzymic control of behaviour, experimental interference with the animal's enzymic activity has been avoided in most of our work. Such studies, correlational in nature, depend upon accurate measurement of normal individual differences in the behaviour of the animal and of normal individual differences in enzymic activity.

Early in our work it became apparent that two important variables which conditioned the relationship between ChE and adaptive behaviour were the age and the genetic background of the animal. In the course of the investigations, therefore, considerable data have been accumulated on individual differences in brain ChE activity and in brain weight in the rat as a function of age and strain.

There are a number of studies in the literature which present growth curves for ChE activity in the brain of the rat (NACHMANSOHN, 1939; METZLER and HUMM, 1951; ELKES and TODRICK, 1955; COHN and RICHTER, 1956). These studies, however, suffer from several defects from our point of view. In most studies the investigator's primary concern was with the first month of life after birth, and the growth curves after that period are usually based upon an exceedingly small number of cases or upon extrapolation. In some studies the number of animals assayed at every age period is so small as to make it worthless to seek any estimate of individual variability. Indeed, in some studies the results of 'two or three animals' are pooled and only the means are reported. In other studies standard deviations as well as the means are given, but these standard deviations are based upon successive measures of the same pooled tissue sample. Thus these standard deviations indicate reliability of the experimenter's measurement techniques, but do not give any information on variability among the experimental animals. Another common attribute of these studies is the failure to report the strain of rat used. Where the strain is specified, no comparable data for another strain are given and so it is impossible to determine the role of genetic factors in brain enzyme activity.

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Quite apart from our own concern with individual differences in brain ChE activity—a concern necessitated by our specific experimental problem—there has been a growing interest in individual differences and variability of biochemical factors in organisms (WILLIAMS, 1956). Because data on individual differences in enzymic activity in the mammalian brain, and specifically ChE activity in rat brain, have rarely been reported in the literature, our data may be of general value.

MATERIALS AND METHODS

The data reported in this paper have been obtained from male rats of two of the strains regularly maintained in the Department of Psychology colony: S_1, descendents of the Tryon Maze-Bright strain (TRYON, 1929) and S_3, descendents of the Tryon Maze-Dull strain. It should be emphasized that these animals are 'descendents,' since TRYON's selective breeding programme of rapid and slow maze learners ended many years ago, and subsequent genetic drift as well as accidental cross-breeding may have changed these strains considerably from their original status. Some of the rats whose data are reported were given behavioural tests prior to sacrifice, others received no such testing. In this paper only the biochemical and brain weight data are presented.

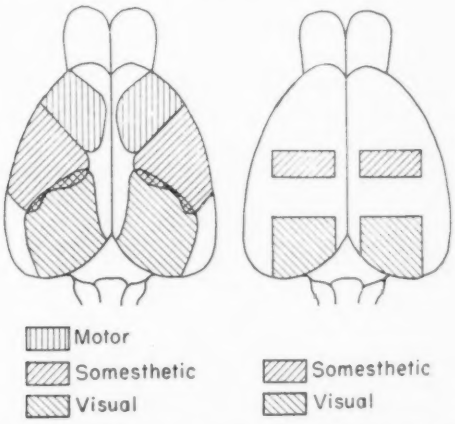

FIG. 1. Diagrams of the dorsal surface of the rat cerebrum. The diagram to the left shows areas mapped by electrophysiological techniques by WOOLSEY and LE MESSURIER (adapted from ZUBEK, 1951). The diagram to the right indicates the cortical areas removed in our work.

When an animal was ready for chemical analysis, it was decapitated, the division being made between the first cervical vertebra and the skull. A total of from 40 to 50 mg of cortical tissue was removed by gross dissection from the visual areas of the two hemispheres, and 25 to 35 mg was removed from the somesthetic areas. These cortical areas are shown in Fig. 1. Since the rat's cortex has no external landmarks, the areas to be removed were determined with the aid of a special plastic T-shaped guide appropriately calibrated. The rest of the dorsal surface of the cortex was then stripped off. The remaining brain, extending from the transection (just behind the cerebellum) through the olfactory bulbs, was kept as a third sample. This sample (i.e. the brain minus the dorsal cortex) will be referred to as 'subcortical brain.' The visual and somesthetic areas, the remainder of the dorsal cortex, and the subcortical brain were weighed rapidly on a projection analytical balance. Thus, when we refer to 'total brain weight' we mean the total wet weight of all of the brain included within the skull.

Originally the samples were analysed the day they were removed, but more recently we have been freezing the samples rapidly on blocks of dry ice and storing them at $-20°C$. The time from decapitation of an animal to placing the samples on dry ice did not exceed 10 min. Storage of samples for periods up to 2 weeks does not appear to affect ChE activity.

An homogenate of the tissue sample was prepared in cold 0·9% NaCl, and the enzymic activity was determined under standardized conditions by the rate of hydrolysis of acetylcholine perchlorate (ACh). The analytical procedure has been reported previously (KRECH, ROSENZWEIG and BENNETT, 1956; ROSENZWEIG, KRECH and BENNETT, 1958). The ChE activity of the tissue sample is reported in terms of moles ACh $\times 10^{10}$ hydrolysed per minute per milligram of tissue. While this analysis does not distinguish between acetylcholinesterase and pseudocholinesterase, it appears unlikely that variations in the pseudocholinesterase activity contribute significantly to the values we have obtained. To measure the pseudocholinesterase activity toward acetylcholine of homogenates of cortical and subcortical brain, we have used a selective inhibitor of acetylcholinesterase, 1:5-bis-(4-trimethylammoniumphenyl)pentan-3-one di-iodide* (BAYLISS and TODRICK, 1956; ELKES and TODRICK, 1955). The results indicate that less than 5 per cent of the activity of the tissue homogenate used can be attributed to pseudocholinesterase.

The primary data thus consist of total brain weight and of three measures of ChE activity: (1) ChE in the visual area of the cortex; (2) ChE in the somesthetic area of the cortex; and (3) ChE in the total brain minus the dorsal surface of the cortex, referred to hereafter as the 'subcortical ChE activity.' Since high correlations have been consistently found between ChE activity in various areas of the cerebral cortex (ROSENZWEIG, et al., 1958), the ChE activities of the visual and somesthetic areas have been averaged to obtain a single index of 'cortical ChE activity.' The three measures of ChE activity are then reduced to two: cortical, and subcortical ChE activity.

TABLE 1.—MEAN, STANDARD DEVIATION AND RANGE OF CORTICAL CHOLINESTERASE ACTIVITY, AS A FUNCTION OF AGE, FOR S_1- AND S_3-STRAINS OF RATS

S_1					S_3				
No.	Age (days)	Mean ChE $\frac{V+S\dagger}{2}$	S.D.	Range	No.	Age (days)	Mean ChE $\frac{V+S*}{2}$	S.D.	Range
3	29	52·2	1·54	50·6–54·3	2	29	49·0	2·40	46·6–51·4
3	34	55·3	1·19	53·8–56·7	4	32	50·5	0·83	49·4–51·6
6	43	61·1	2·80	55·8–64·8	9	43	57·2	3·89	54·0–67·6
9	62	64·2	2·84	61·4–69·2	8	64	58·2	3·15	53·8–63·1
9	82	63·0	3·06	58·0–68·4	7	81	60·6	4·60	50·6–64·8
27	96	63·5	4·35	55·0–75·0	14	96	57·7	3·28	53·6–65·0
50	108	65·3	3·95	58·6–72·4	54	110	59·6	3·47	52·7–69·0
56	118	62·8	3·16	56·2–69·7					
50	128	64·4	3·83	52·0–72·8	41	126	57·2	3·45	51·2–63·6
					17	131	60·0	2·92	55·3–64·6
13	147	61·8	3·95	55·0–73·0	6	147	56·5	2·14	52·4–58·4
					3	171	56·7	5·73	50·0–64·0
5	245	63·9	3·06	59·2–68·4	6	244	55·3	2·14	51·3–58·0
					11	260	58·2	5·49	51·0–67·0
8	370	60·4	4·32	55·7–68·0	3	376	58·1	2·73	54·2–60·1
2	500	58·5	2·50	56·0–61·0	6	527	55·0	1·29	53·0–57·0

† Cholinesterase activity was averaged for samples of visual and somesthetic regions of cerebral cortex and is expressed in moles ACh $\times 10^{10}$ hydrolysed per min per mg of tissue.

RESULTS

Cortical cholinesterase activity. Table 1 presents the data for cortical ChE activity for rats of the S_1- and S_3-strains from 29 to 500 days of age. During 3 years of analytical work, data have been obtained for 241 S_1-rats and 191 S_3-rats. In preparing the

* Dr. D. W. ACKERMAN of the Wellcome Research Laboratories, Beckenham, Kent, England, kindly provided us with a sample of 1:5 bis-(4-trimethylammoniumphenyl)pentan-3-one di-iodide.

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table, data have been grouped together from all animals sacrificed within a 10 day interval of age, each interval starting with an integral multiple of 10; i.e. 20–29 days, 30–39, 40–49, and so on. For each age group the mean age, and the mean standard deviation and range of the ChE values were computed. Certain groups were very close to each other in age and ChE values, and therefore the number of groups was reduced by combining adjacent ones when their mean ages did not differ by more than 10 per cent and when their mean ChE values did not differ significantly at the 5 per cent level of confidence. When groups were combined, the tabulated statistics represent the whole group.

The changes in cortical ChE activity with age are shown graphically in Fig. 2.

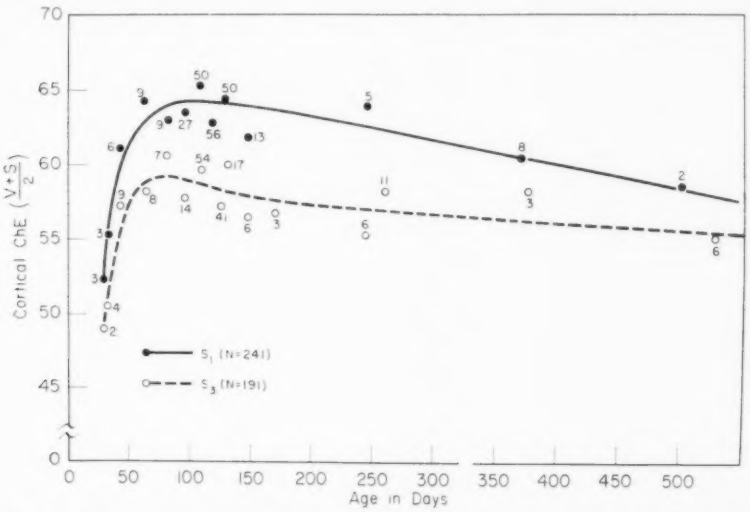

FIG. 2. Cortical cholinesterase (ChE) activity of the S_1- and S_3-strains of rats as a function of age. The ChE values are based on an average of samples obtained from the visual and somesthetic areas of the cortex. They are expressed in moles ACh $\times 10^{10}$ hydrolysed per min per mg of tissue. The number at each point shows the number of animals upon which the value is based.

The ChE values for both strains rise sharply until about 60 days, and then they continue upward more gently, reaching a broad peak in the region of 100 days. COHN and RICHTER (1956) reported that the peak is reached at 60 days, but a personal communication from Dr. COHN makes it clear that they used few animals older than 60 days. After 100 days our values decline slowly and continuously, subsiding at 500 days to about the level they had at 40 days.

This decline after 100 days, it should be remembered, is in ChE activity per unit of tissue weight. Since the weight of the brain continues to grow steadily (Fig. 4), this does not necessarily imply that total ChE activity of the cortex declines. We had available the weight of the dorsal cortex for animals up to 130 days of age and could therefore determine total cortical ChE activity [(cortical ChE activity per unit weight) $\times$ (cortical weight)]. These determinations indicate that total cortical ChE activity remains essentially constant after 100 days.

At every age the mean ChE values for the S_1-strain are higher than those for the S_3-strain, the mean difference being about 10 per cent. An analysis of variance was

done in order to determine whether the difference in ChE between strains is statistically significant. Since the numbers of cases in the various subgroups differ widely, the technique of analysis of variance for disproportionate subclasses was used (SNEDECOR, 1956). The difference between the two strains was found to be highly significant (1 and 387 *df*; $F = 181{\cdot}5$; $P < 0{\cdot}001$). The variance due to differences in age was also highly significant (15 and 387 *df*; $F = 12{\cdot}3$; $P < 0{\cdot}001$). The interaction was not significant, indicating that the *shapes* of the growth curves cannot be distinguished for the two strains.

TABLE 2.—MEAN, STANDARD DEVIATION AND RANGE OF SUBCORTICAL CHOLINESTERASE ACTIVITY, AS A FUNCTION OF AGE, FOR S_1- AND S_3-STRAINS OF RATS

S_1					S_3				
No.	Age (days)	Mean ChE	S.D.	Range	No.	Age (days)	Mean ChE*	S.D.	Range
5	28	158·2	8·33	144–169	4	29	150·0	7·58	142–159
3	34	153·7	3·09	151–158	4	32	153·0	2·12	150–155
8	44	165·4	3·50	161–173	9	43	165·2	8·51	153–184
9	62	170·3	8·41	154–186	8	64	160·1	5·88	150–167
7	80	178·4	8·91	163–199	7	81	169·3	9·05	159–185
25	104	167·9	9·72	154–192	19	98	163·1	9·28	148–182
38	114	161·8	9·16	137–180	32	114	154·8	11·91	134–180
45	128	167·3	7·65	149–182	35	127	151·2	6·76	140–164
					17	131	157·4	8·59	142–171

* Cholinesterase activity is expressed in moles ACh $\times 10^{10}$ hydrolysed per min per mg of tissue.

The statistics on variability (the standard deviation and range) make it possible to characterize the variability among individual rats in cortical ChE activity. The relative variability can be expressed by the coefficient of variation [$V = (100 \text{ S.D.})/\text{mean}$]. When V is computed for each of the subgroups, the average value for the S_1-rats is 5·70 and for the S_3-rats, 5·73. These coefficients indicate that the individual values tend to cluster rather closely around the means of their groups, two-thirds of the animals having values that differ by less than 5·7 per cent from the mean of their group.

Subcortical cholinesterase activity. Table 2 presents the data for subcortical ChE activity for the two strains. Subcortical ChE activity has been analysed only during the past year and a half, and values are given for 140 S_1-rats and 135 S_3-rats, distributed over the age range of from 28 to 131 days. The data were grouped according to age and combined as in the case of the cortical ChE data.

The age functions of subcortical ChE activity are shown in Fig. 3. The curves for both strains show rather sharp peaks at about 80 days. This is in keeping with the finding reported by several workers (ELKES and TODRICK, 1955; COHN and RICHTER 1956) that subcortical centres reach their maximum ChE activity at an earlier age than does the cortex. (Again the drop from the maximum is found only when the measure is of ChE activity per unit of tissue weight. Total subcortical ChE activity remains essentially constant after 80 days.) Overall, the S_1-values exceed the S_3-values by

about 6 per cent. An analysis of variance for disproportionate subclasses was performed to test the significance of the observed differences. The difference in favour of the S_1-strain was found to be highly significant (1 and 258 *df*; $F = 66{\cdot}7$; $P < 0{\cdot}001$). The variance due to differences in age was also significant (8 and 258 *df*; $F = 8{\cdot}0$; $P < 0{\cdot}001$). The interaction was also significant, the two strains differing more at the later than at the earlier ages (7 and 258 *df*; $F = 3{\cdot}0$; $P < 0{\cdot}01$).

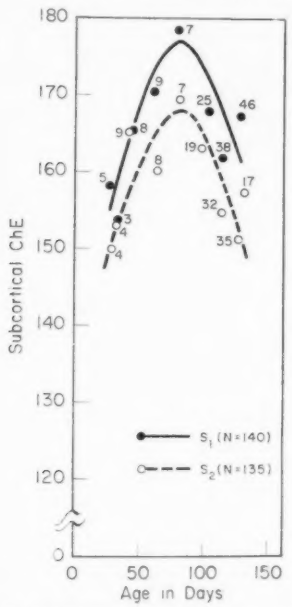

FIG. 3. Cholinesterase (ChE) activity in the subcortical brain of the S_1- and S_3-strains of rats as a function of age. The ChE values are for brains from which dorsal cortex has been removed. They are expressed in moles ACh $\times 10^{10}$ hydrolysed per min per mg of tissue. The number at each point shows the number of animals upon which the value is based.

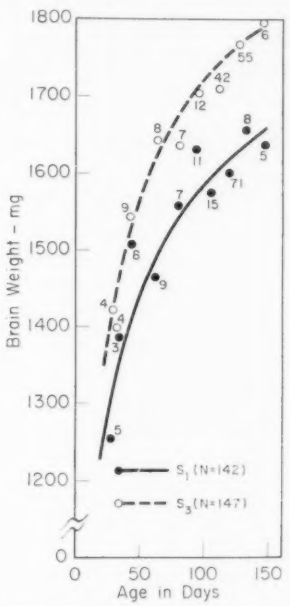

FIG. 4. Brain weight of the S_1- and S_3-strains of rats as a function of age. The entire brain was used, including the cerebellum and the olfactory bulbs.

The coefficients of variation tend to be somewhat smaller for subcortical than for cortical ChE activity. They average 4·42 for the S_1-groups and 4·88 for the S_3-groups. Thus the rats show even less relative variability in subcortical than in cortical ChE activity.

Brain weight. Table 3 presents weights of the total brain for rats of the two strains. Brain weights are available for 142 S_1-rats and 147 S_3-rats, distributed through the age range of from 28 to 148 days. Data were grouped and combined as in the case of the ChE data.

The age functions in Fig. 4 rise monotonically. Contrary to the case of the ChE activity data, here it is the S_3-strain whose values are the greater at every age tested. An analysis of variance, done for disproportionate subclasses, showed that the strain difference was again highly significant (1 and 260 *df*; $F = 224{\cdot}4$; $P < 0{\cdot}001$). The age differences were also highly significant, as would be expected for sharply rising functions (9 and 260 *df*; $F = 40{\cdot}8$; $P < 0{\cdot}001$). The interaction is not significant,

the vertical distance between the curves for the two strains increasing only slightly with age.

The coefficients of variation are somewhat smaller for brain weight than for the ChE activity measures. They average 3·80 for brain weights of the S_1-strain and 4·45 for the S_3-strain.

TABLE 3.—MEAN, STANDARD DEVIATION AND RANGE OF BRAIN WEIGHT, AS A FUNCTION OF AGE, FOR S_1- AND S_3-STRAINS OF RATS

S_1					S_3				
No.	Age (days)	Mean weight (mg)	S.D.	Range	No.	Age (days)	Mean weight (mg)	S.D.	Range
5	28	1255·0	44·4	1197–1310	4	29	1422·2	49·2	1380–1502
3	34	1386·0	36·1	1347–1434	4	32	1398·8	46·4	1325–1453
8	44	1506·8	60·0	1403–1563	9	43	1542·7	55·3	1453–1620
9	62	1464·8	58·8	1357–1582	8	64	1642·4	93·8	1516–1746
7	80	1557·9	65·0	1480–1644	7	81	1635·8	116·2	1439–1803
11	94	1630·9	73·3	1510–1770	12	96	1704·5	70·6	1601–1840
15	105	1575·6	55·6	1436–1644	42	112	1710·6	72·0	1562–1842
71	120	1600·1	65·4	1439–1736	55	128	1766·8	82·5	1600–1940
8	133	1657·1	63·7	1570–1770					
5	148	1638·2	61·6	1543–1721	6	147	1796·5	69·0	1657–1859

DISCUSSION

It is clear from the results that differences in brain chemistry and brain weight are associated with differences in the age and strain of rats. The analyses of variance demonstrated the statistical significance of these sources of variance. It is perhaps worth considering whether the differences among animals of a given age and strain are also significant or whether they may be attributed to errors of measuring brain weight and ChE activity.

The brains are weighed with less than 10 mg of error, the error being due primarily to differential loss of water. Tests have shown that variations in techniques of removal of the brain could account at the most for 20 mg of error. Such errors could certainly not account for the differences of 300 mg or more found between brains of rats of the same strain and age; therefore it is clear that the observed within-group differences are primarily due to true individual differences.

Two ChE determinations are usually made on each tissue sample, and the mean is taken. Differences between the two analyses of a given tissue sample average about 2 units of ChE activity for the cortex and 4 units for the subcortex; taking the average of the two determinations for each of these parts of the brain further reduces the error of measurement. Such errors could not account for differences among rats of the same age and strain of 20 units at the cortex or of 40 units in the subcortical brain. The conclusion that there are real individual differences in brain ChE activity is given further support from a genetic experiment in progress in which it has been possible to breed new strains of rats for high and low cortical ChE activity by selecting their sires according to our standard ChE determination techniques (RODERICK, BENNETT

and KARLSSON, 1957). Finally, there is the fact that statistically significant correlations have been obtained between our chemical measurement and behavioural measurements within strains. This is further evidence that the individual differences in brain chemistry (and in behaviour) are reliable; if they were not, the correlations would reflect only random variation, and would not differ significantly from zero.

The variability of the brain is small compared with the variability of other organs. JACKSON (1913) found that the coefficient of variation for brain weight of the rat was smaller than that for any of the thirteen other organ or system weights he measured. Our data indicate that the enzymic variability of the brain is also under strict control, for the coefficients of variation for ChE activity are only slightly larger than those for brain weight. We have also found comparable coefficients of variation for lactic dehydrogenase activity in the rat brain. Since individual variation in brain chemistry is small, though significant in magnitude, great care is necessary in making determinations that are to measure values for individual subjects.

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The shape of the curve for ChE activity of the subcortical brain should approximate that for the whole brain. These values, it will be recalled, were determined after the dorsal cortex—about 20 per cent of the weight of the entire brain—was removed. Since the ChE activity of the dorsal cortex is about 40 per cent of that of the subcortical brain, the level of the curve for subcortical brain should be reduced by about 12 per cent to give approximate values for the whole brain. The most extensive curve in the literature on ChE development in the rat brain is that of METZLER and HUMM (1951) for the 'white rat.' It extends from the 14-day foetus to the 'adult'; there are fifty-one points (presumably individual rats) over the whole age range. Only thirteen points are for ages greater than 28 days, the age at which our curve begins. The curve of METZLER and HUMM shows an abrupt peak at 22 days, then a decline to a plateau which extends from 32 to 75 days and another decline to the 120 day and 'adult' level. ELKES and TODRICK (1955) studied development of ChE activity in the whole brain of the 'albino rat.' They used fifty rats distributed at 3 or 4 day intervals between the third and thirtieth day of life. Their curve is monotonic and shows no sign of the 22 day peak of METZLER and HUMM. They state that the level at 22 days is approximately 75% of the fully grown level. NACHMANSOHN (1939), using single rats for each value, found a rise in ChE activity between 21 and 35 days, a smaller rise from 35 to 110 days, and then a decline from 110 to two 'adult' animals. BAYLISS and TODRICK report in an abstract (1956) that ChE activity increases linearly from the third to the twentieth day and more slowly thereafter. It may be that different strains of rats differ considerably in growth curves of brain ChE activity. However, in the light of all the evidence, we are inclined to believe that ChE activity for whole brain of the rat reaches its peak around the eightieth day of life.

The change of ACh content with age in whole brain is asserted by WELSH and HYDE (1944) to be closely correlated with that of ChE activity. They, and CROSSLAND (1951), found that ACh doubles from 24 hours to 21 days and doubles again when the rat is '100+' days old. While the direction of change is the same for ACh and ChE, it appears that ChE activity increases more rapidly than ACh up to 21 days and then more slowly than ACh.

Our ChE activity determinations, like those of others, give activity per unit of weight. It is interesting to note that the S_3-strain has equal or slightly larger total ChE activity than the S_1-strain (ChE activity $\times$ brain weight). Since the S_3-brains are

heavier, this activity is less per unit of weight in the S_3-brain. It is possible that the ChE difference reflects a different ratio of neural to glial structures in the two strains. Such an anatomical difference between strains could have important functional significance. If such an anatomical difference exists, it cannot be uniform throughout the brain, since the ChE difference is relatively larger at the cortex than subcortically. An histological investigation to attempt to determine the source of the strain differences in ChE activity is in progress.

SUMMARY

Cholinesterase (ChE) activity in the cortical and subcortical brain, and brain weight, were determined for male rats of two strains as a function of age. The S_1-strain was found to have significantly greater cortical and subcortical ChE activity and significantly lower brain weight than the S_3-strain. Significant effects of age were also found for each variable. It was shown that for animals of a given strain and age, there are true individual differences that cannot be attributed to errors of measurement. Thus the rat brain ChE activity and brain weight differ according to genetic background, age and individual factors.

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CHOLINESTERASE AND LACTIC DEHYDROGENASE ACTIVITY IN THE RAT BRAIN*

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PREVIOUS studies have demonstrated the existence of reliably measurable individual, strain and age differences in cholinesterase (ChE) activity of the rat brain (BENNETT, ROSENZWEIG, KRECH, KARLSSON, DYE and OHLANDER, 1958). These individual differences in ChE activity have also been shown to be related to the adaptive behaviour of the rat (ROSENZWEIG, KRECH and BENNETT, 1958). This latter relationship has been assumed to reflect the fact that the acetylcholine-ChE system is of peculiar significance in the metabolism of the brain because of its contribution to synaptic transmission. However, since the acetylcholine-ChE system is only one of the numerous biochemical systems which are important in the metabolism of the brain, the correlations which have been found between ChE activity of the cortex and the behaviour of the rat may be non-specific; that is, ChE activity may simply reflect the *general* enzyme and metabolic levels in the rat brain. It is therefore of interest to determine what relationships exist between ChE and other enzyme systems. In this paper ChE activity is compared with lactic dehydrogenase (LDH) activity in the cerebral cortex and subcortical brain of the strains of rats we have used in many of our previous studies.

Lactic dehydrogenase carries out the interconversion:

$$\text{pyruvic acid} \rightleftharpoons \text{lactic acid}$$

which is the last step in the glycolytic formation of lactic acid from glucose.

Normally, the brain oxidizes about 85 per cent of the glucose it utilizes, while it metabolizes only about 15 per cent of the glucose by the glycolytic route to lactate (MCILWAIN, 1955). However, during periods of maximal nerve activity, the glycolytic pathway may assume increased importance. Therefore, if it is assumed that high levels of ChE are associated with a potentially high general activity of the brain, a positive correlation might be expected between ChE and LDH activity. On the other hand, the same assumption that high levels of ChE are associated with a potentially high general activity of the brain might lead one to expect an inverse relationship between ChE and LDH. This would follow from the argument that the formation of

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lactic acid from glucose represents less efficient utilization of glucose than does the complete oxidation via the tricarboxylic acid cycle. Thus the less adaptive rats with low ChE activity in the cortex would be expected to have high LDH activity representing the less efficient utilization of energy sources. Finally, it is possible that the two systems are quite unrelated. If no correlation were found to exist between LDH and ChE this would support the hypothesis that the observed correlations between cortical ChE activity and adaptive behaviour did not reflect a correlation between general metabolic rate in the brain and adaptive behaviour, but, rather, was specific to the acetylcholine–ChE cycle.

METHODS

The LDH and ChE activities were determined for the visual (V) and somesthetic (S) areas of the cerebral cortex and for the subcortical brain (brain with dorsal cortex removed). To give a single value for cortical enzyme activity, the determinations for the V and S areas were averaged [$(V + S)/2$]. Fifty-nine male rats of the S_1-strain and forty-seven male rats of the S_3-strain were tested. These rats were descendants of the two strains selectively bred by TRYON (1929) for high and low maze-learning ability. They range in age from 30 to 148 days. These animals were included among 432 rats whose ChE values were reported in a previous paper (BENNETT *et al.*, 1958).

Lactic dehydrogenase activity was estimated by the spectrophotometric measurement at 340 mμ of the rate of formation of reduced diphosphopyridine nucleotide (DPNH) (NEILANDS, 1955). The assay mixture consisted of 2 ml of 0·1 M-glycine buffer, pH 9·2; 0·5 ml M-sodium DL-lactate, pH 9·2; and an aliquot portion of the same tissue sample homogenate used for the ChE determination (50 μl of the homogenates containing 100 μg wet weight of the cortex, or 100 μl of a one-tenth dilution of the subcortical brain homogenates containing 150 μg of tissue was used). The mixture was pre-incubated in 1 cm Beckman cells at 37° in a thermostatically controlled cell compartment of a Beckman DU spectrophotometer. After 10 min, 0·4 ml of DPN (10 mg/ml, Sigma 95–100% pure) was added to each sample, and the solutions were mixed and replaced in the cell compartment of the spectrophotometer. After 3 min when temperature equilibrium was obtained, the optical density was determined at 340 mμ for 20 min, at 5 min intervals. The change of optical density/min was calculated by the formula:

$$\Delta \text{ OD/min} = \frac{N\Sigma XY - (\Sigma X)(\Sigma Y)}{N\Sigma X^2 - (\Sigma X)^2}$$

where X is time, and Y is the density reading. The values for LDH activity have been expressed in terms of moles of DPN reduced $\times 10^9$ per min/mg wet tissue, using 6280 as the molecular extinction coefficient for DPNH (HORECKER and KORNBERG, 1948).* Duplicate analyses were made of each sample and the results averaged.

Cholinesterase activity was determined by the rate of hydrolysis of acetylcholine in an automatic titrator. The methods have been reported in detail (ROSENZWEIG, KRECH and BENNETT, 1958).

RESULTS

The results of the analyses of the two enzymes are summarized in Tables 1 and 2 and Fig. 1. They show several significant differences between the ChE and LDH activities in the rat brain:

(1) The distribution of LDH in the cortex and subcortex is quite unlike that of ChE. We have consistently found with both strains that the ChE activity of the V-section of the cortex is 15 to 20 per cent less than that of the S-section (see Fig. 1), and the average ChE activity of the V and S sections is only 35 to 40 per cent of the activity in the subcortical brain. These findings are repeated with the present sample. On the

* Preliminary experiments indicated that the change in specific activity of LDH was less than 3 per cent with a 25 per cent change in the weight of samples used in the range of from 50 to 150 μg of cortex. Less than 10 per cent change in specific activity was observed when the amount of DPN added was varied from 2 to 8 mg or the volume of 0·5 M-lactate added was varied from 200 μl to 600 μl.

other hand, the present data show that the LDH activity of the *V*-section is the same or slightly higher than that of the *S*-section, and the averaged LDH activity of the *V*- and *S*-sections is from 110 to 120 per cent of the activity in the subcortical brain (Tables 1 and 2).

TABLE 1.—COMPARISONS BETWEEN CHOLINESTERASE AND LACTIC DEHYDROGENASE ACTIVITIES IN BRAIN OF S_1-RATS AT SIX DIFFERENT AGES

			Cholinesterase activity*				Lactic dehydrogenase activity†			
Age (days)	No.		*V*	*S*	$\frac{V+S}{2}$	Sub-cortex	*V*	*S*	$\frac{V+S}{2}$	Sub-cortex
30	6	$\bar{X}$	48·1	59·5	53·8	153·3	77·4	73·8	75·5	63·9
		S.D.	2·89	1·54	2·07	5·09	2·23	1·09	1·62	1·19
		Range	43·9–51·9	57·4–61·6	50·6–56·7	144–159	75·6–81·7	72·8–76·1	74·2–78·9	63·1–66·4
44	6	$\bar{X}$	55·9	66·4	61·1	165·8	75·0	74·5	74·8	65·1
		S.D.	1·73	4·40	2·80	3·89	2·86	3·25	1·01	0·90
		Range	53·0–58·1	57·5–71·6	55·8–64·8	161–173	72·3–80·8	69·1–78·0	73·3–76·6	64·0–66·9
92	6	$\bar{X}$	57·6	65·6	61·6	‡	69·8	69·6	69·7	‡
		S.D.	1·75	1·97	1·74		2·53	1·63	1·95	
		Range	54·0–59·3	62·1–68·1	58·0–63·3		65·8–71·9	67·2–72·3	66·7–71·9	
110	20	$\bar{X}$	59·2	69·2	64·2	163·3	72·2	73·6	72·9	64·3
		S.D.	3·21	3·39	3·12	11·28	3·64	2·98	2·33	3·08
		Range	55·1–67·2	63·2–76·3	59·8–71·8	173–192	67·6–81·3	67·2–78·0	68·1–78·4	57·8–69·7
129	15	$\bar{X}$	58·3	65·4	61·9	171·1	77·7	72·5	75·2	65·7
		S.D.	2·46	3·22	2·47	6·40	1·66	4·36	2·40	1·98
		Range	54·0–63·7	58·2–70·3	56·8–65·5	158–182	75·6–80·8	66·7–80·8	70·9–79·4	62·1–69·7
147	6	$\bar{X}$	57·5	64·6	61·1	‡	69·3	70·0	69·7	‡
		S.D.	1·64	2·67	1·61		2·57	1·49	1·89	
		Range	54·6–59·6	60·5–69·1	59·0–64·2		65·8–72·8	68·6–71·9	67·2–72·3	

* Expressed in terms of moles × 10^{10} acetylcholine hydrolysed/min/per mg tissue.
† Expressed in terms of moles × 10^9 DPNH formed per min/mg tissue.
‡ Subcortical values are not available for these groups.

(2) The effect of age differs upon ChE and LDH activity. ChE activity per unit of weight of cortex has been shown to increase with the age of the rat to a maximum at about 100 days; thereafter the activity declines slowly (BENNETT *et al.*, 1958). The early increase is reflected in Tables 1 and 2 and in Fig. 1; the tables do not include animals old enough to show the decline in ChE at later ages. On the contrary, the

LDH activity of the cortex is essentially the same at all the ages tested, as can be seen from Tables 1 and 2. (For subcortical brain, not enough values are available for both ChE and LDH to permit determinations of comparative age functions.)

TABLE 2.—COMPARISONS BETWEEN CHOLINESTERASE AND LACTIC DEHYDROGENASE ACTIVITIES IN BRAIN OF S_3-RATS AT FIVE DIFFERENT AGES

			Cholinesterase activity*				Lactic dehydrogenase activity†			
Age (days)	No.		V	S	$\frac{V+S}{2}$	Sub-cortex	V	S	$\frac{V+S}{2}$	Sub-cortex
31	6	$\bar{X}$	43·3	56·8	50·0	149·5	75·8	75·1	75·5	63·2
		S.D.	1·68	3·09	1·71	5·25	2·99	3·09	0·89	1·67
		Range	41·4–46·5	51·4–60·7	46·6–51·6	142–155	71·4–79·9	69·5–78·0	74·2–77·0	61·1–65·4
94	6	$\bar{X}$	51·0	63·1	57·0	‡	71·4	69·9	70·6	‡
		S.D.	2·68	2·43	2·35		0·54	2·46	1·04	
		Range	47·1–54·8	60·1–66·0	53·6–60·0		70·5–71·9	67·2–74·2	69·1–72·3	
108	3	$\bar{X}$	52·2	61·3	56·7	166·3	70·8	69·5	70·1	64·3
		S.D.	0·31	3·50	1·94	8·34	2·93	1·67	1·45	0·22
		Range	51·9–52·6	57·8–66·1	54·8–59·4	159–178	67·6–74·7	68·1–71·9	68·1–71·4	64·0–64·5
128	26	$\bar{X}$	51·0	61·6	56·3	150·7	68·9	75·5	71·2	61·7
		S.D.	2·62	3·70	2·87	6·37	3·55	3·58	2·51	3·02
		Range	45·8–55·1	55·4–72·4	51·2–62·7	141–161	62·0–76·1	67·6–80·8	65·8–75·6	54·5–66·4
148	6	$\bar{X}$	51·4	61·7	56·5	‡	69·7	69·4	68·5	‡
		S.D.	1·73	2·89	2·14		2·44	1·47	1·55	
		Range	48·7–54·2	56·1–65·1	52·4–58·4		66·2–74·2	66·7–70·9	66·7–70·8	

* Expressed in terms of moles $\times 10^{10}$ acetylcholine hydrolysed/min/per mg tissue.
† Expressed in terms of moles $\times 10^{9}$ DPNH formed/min/per mg tissue.
‡ Subcortical values are not available for these groups.

(3) ChE and LDH differ in relations between strains. We have previously reported (BENNETT *et al.*, 1958) that the variability of ChE activity is approximately 5 per cent when strain, brain area and age are held constant. A similar result for the variability of LDH activity is indicated by the small values of the standard deviation in relation to the means and by the ranges (Tables 1 and 2). This is further evidence for the low biochemical variability of the brain as compared with other tissues. Despite this low variability, we have consistently found a statistically significant difference between the ChE levels of the S_1 and S_3 strains such that the S_1-strain has always been higher. This superiority of the ChE level of the S_1-strain is found at every age and for every brain area measured (see Fig. 1). However, no such strain differences

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are found for LDH activity. As can also be seen from Fig. 1, the LDH activity per unit weight is very much the same for both the S_1 and the S_3-strains. This is true for every age and for every brain area tested.

When, however, we consider *total* enzyme activity instead of activity per unit weight, a different picture emerges. The brain weight of the S_3-strain is significantly

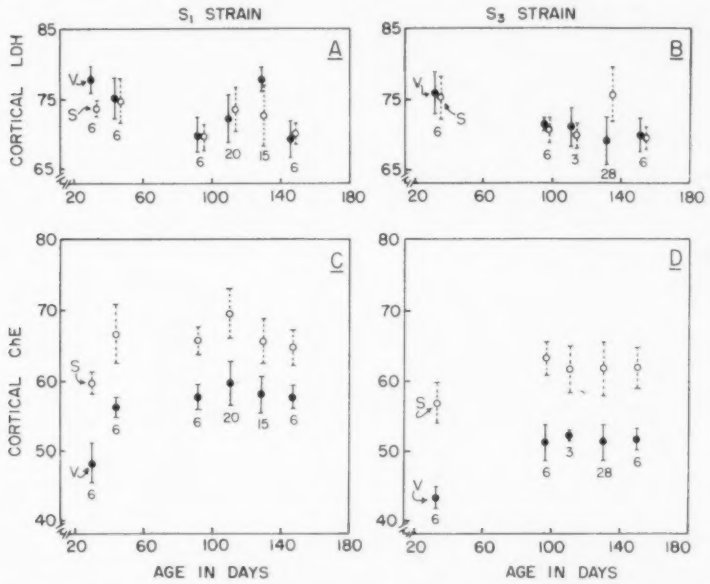

FIG. 1.—Boxes A and B give cortical lactic dehydrogenase activity for the S_1- and S_3-strains, respectively. The LDH is expressed in terms of moles $\times 10^9$ DPNH formed/min/per mg tissue. The mean LDH activity of the visual area (*V*) of the cortex is shown by a solid dot, the upper and lower ends of the solid bar indicating $\pm$ one standard deviation from the mean. The LDH activity of the somesthetic area (*S*) is similarly shown by an open dot and a dotted bar. The number of animals is indicated for each age. Since the LDH values for visual and somesthetic areas overlap, the visual values have been displaced slightly to the left and the somesthetic values slightly to the right on the graph. Boxes C and D give corresponding values for cortical cholinesterase activity expressed in terms of moles $\times 10^{10}$ ACh hydrolysed/min/per mg tissue.

greater than that of the S_1-strain (BENNETT *et al.*, 1958), and brain weight of the rat increases steadily with age. When total activity is calculated, taking into account the brain weight, the S_1-strain shows a higher total ChE activity than the S_3-strain in the cortex but a lower total in the subcortex. For LDH, on the other hand, the S_1-strain shows a lower level total activity than the S_3-strain in both the cortex and the subcortex.

(4) The generality within the brain of ChE and LDH activity differs significantly. As shown in Table 3, fairly high, statistically significant and positive correlations exist between ChE activity in the *V*-sections of the cortex and in the *S*-sections. This holds for either strain. The size of the overall correlation, 0·67, indicates that this relationship accounts for 45 per cent of the variance; that is, knowing the ChE value for one cortical region of any animal, the uncertainty in the value for the other region is reduced by 45 per cent. In previous work (ROSENZWEIG, KRECH and BENNETT, 1958), it has been shown that the ChE activity in the *V* and in the *S*-sections is also highly correlated with ChE activity in the motor section of the rat's cortex. Thus it appears

TABLE 3.—CORRELATIONS BETWEEN AND AMONG CHOLINESTERASE AND LACTIC DEHYDROGENASE ACTIVITY

Correlations among cholinesterase values							Correlations among lactic dehydrogenase values						
Age (days)	Strain	No.	V vs. S	V vs. subcortex	S vs. subcortex	$\frac{V+S}{2}$ vs. subcortex	Age (days)	Strain	No.	V vs. S	V vs. subcortex	S vs. subcortex	$\frac{V+S}{2}$ vs. subcortex
110	S_1	20	0·75‡	0·42	0·15	0·30	110	S_1	20	0·04	0·17	−0·20	0·04
129	S_1	15	0·54†	0·03	0·37	0·26	129	S_1	15	0·03	−0·17	−0·46	−0·50
128	S_3	26	0·67‡	0·37	0·17	0·28	128	S_3	26	−0·05	−0·04	0·26	0·17
	Both str. combined§	61	0·67‡	0·31*	0·21	0·28*		Both str. combined	61	0·00	0·00	−0·07	−0·04

Correlations between cholinesterase and lactic dehydrogenase values

Age (days)	Strain	No.	V vs. V	S vs. S	Subcortex vs. subcortex	$\frac{V+S}{2}$ vs. $\frac{V+S}{2}$
110	S_1	20	0·56†	−0·05	−0·04	0·37
129	S_1	15	−0·04	0·07	0·35	0·07
128	S_3	26	0·15	0·35	−0·21	0·51†
	Both str. combined	61	0·26	0·16	−0·22	0·37†

* Significant at 0·05 level.
† Significant at 0·01 level.
‡ Significant at 0·001 level.
§ Correlations for the combined strains are weighted averages using FISHER's r to z transformation.

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that the level of the ChE activity in the rat cortex is a *general* characteristic of the cortex. Table 2 also shows that there is a positive, but considerably lower, correlation between the ChE activity in the cortex and in the subcortical brain. Here, however, none of the correlations for either strain is significantly greater than zero. However, the correlation for both strains combined (as indicated in the table) is significant at the 5 per cent level of confidence. The corresponding correlational picture for LDH is radically different. The LDH activity in the *V*-section does not correlate significantly with that in the *S*-section, nor do the cortical values correlate significantly with the subcortical ones. The correlations which do appear are not consistent in sign as we go from one age group to another, or from one strain to another. In other words, the level of LDH activity at one locus of the brain seems unrelated to the activity at other loci.

(5) There appears to be a moderate positive relationship between the ChE and LDH activity levels of the cerebral cortex (see lower right-hand column in Table 3). Although only one of the three subgroups shows a significant relationship, the correlation for all groups combined is significant at the 1 per cent level of confidence. The size of the correlation, 0·37, indicates that this relationship accounts for 14 per cent of the variance. In the subcortical brain, however, there is no consistent relationship between ChE and LDH levels. It appears, therefore, that whatever metabolic process is reflected by the level of ChE activity it is somewhat related in the cortex to the process governed by LDH but the two processes are unrelated in the rest of the brain.

DISCUSSION

The results of the present study clearly indicate that the distribution of LDH and ChE activities in the cortex and subcortical brain of the rat differ in many significant respects. Of particular interest, in the light of our major programme of investigating the enzymatic control of behaviour, is the low correlation between the ChE and LDH levels within the individual rat, and the lack of any difference in LDH levels between the S_1 and S_3-strains. The low correlation suggests that while the ChE level of the rat brain is somewhat related to metabolic systems quite removed from the acetylcholine–ChE system, most of the variance (about 85 per cent) is independent of LDH. To this extent, then, the observed correlations between ChE and adaptive behaviour cannot be ascribed to some *general* metabolic level. The fact that there is no difference between the LDH levels of the S_1 and S_3-strains, while there is a difference between the ChE levels of the two strains, further supports this conclusion. In almost every behavioural test we have used thus far, the S_1-strain has been consistently superior to the S_3-strain. Thus, as far as strain comparisons are concerned, we find a relationship between ChE and performance on our behavioural tests, but no relationship between LDH and performance.

Quite aside from this consideration, however, analysis of the cyto-architectural distribution of brain enzymes supports the general conclusion of a large degree of independence between ChE and LDH. The rat cortex is a complex of cell types and layers, the proportions of which change with the age of the animal and the areas of the cortex. Pope (1952) has shown that the ChE activity of the rat somesthetic cortex varies from layer to layer, generally decreasing by a factor of 2 from layer I to layer VI and the underlying white layer. This suggests the ChE is localized at the surfaces of dendrites and axones including synaptic terminals.

KUHLMAN and LOWRY (1956) have studied the changes in concentration of four dehydrogenases in the cortex of the newborn, the 10 day old, and the adult rat. Lactic dehydrogenase concentration, unlike that found by POPE (1952) for ChE, increases from layer I to layer II, and then remains fairly constant through layer VII.

These observations also suggest that some of the differences we have observed between the activity levels of the two enzymes, ChE and LDH, as well as the differences in ChE between the two strains, may be related to variations in architectonic structure of the cortex. Further investigations on this problem are now in process.

SUMMARY

Cholinesterase (ChE) and lactic dehydrogenase (LDH) activities were determined for 106 male rats of two strains and several ages. Both enzymes show low inter-individual variability. The values for the two enzymes differ in the following respects:

(1) ChE activity shows a more sharply differentiated pattern of regional distribution within cortex and between cortex and subcortex than does LDH activity.

(2) ChE shows a clear rise from 30 to 100 days between our strains while LDH does not.

(3) ChE shows significant strain differences while LDH does not.

(4) ChE values are significantly correlated from locus to locus within the brain, while LDH values are independent from locus to locus.

(5) ChE and LDH activity show some correlation with each other in the cortex but not in the subcortex.

These results are interpreted to mean that in the previously observed correlations between ChE activity and behaviour, ChE activity is not simply an index to general metabolic level of the brain.

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DETERMINATION OF GLUTAMIC ACID, GLUTAMINE, GLUTATHIONE AND γ-AMINOBUTYRIC ACID AND THEIR DISTRIBUTION IN BRAIN TISSUE

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SOME time ago a method for the micro determination of glutamic acid* and glutamine in tissues was reported from this laboratory (PRESCOTT and WAELSCH, 1946; BESSMAN, MAGNES, SCHWERIN and WAELSCH, 1948). Glu and Gline were separated on alumina columns, Glu itself and that produced from Gline converted into the semialdehyde of succinic acid, and the aldehyde as the dinitrophenylhydrazone determined colorimetrically in alkaline solution. GSH was removed on the column by reaction with lead carbonate. This method which, in our laboratory and that of others, had proved to be sensitive and accurate, was on the other hand relatively cumbersome and did not determine Gaba and GSH, two compounds of interest in the study of Glu metabolism in the nervous system.

We wish to report a micromethod for the determination of Glu, Gline, GSH and Gaba on aliquots of protein-free filtrates. The four compounds are separated on columns, converted into γ-lactones by treatment with nitrous acid and then to their corresponding hydroxamic acids (cf. SACHS and BRAND, 1954). The colour developed with the ferric chloride reagent is measured. With this method the four compounds may be determined in duplicate in samples of brain tissue as small as 100 mg, or if only Glu and Gline determinations are desired, in about one-tenth of this amount of tissue.

METHOD

Separation of Glu, Gline, GSH and Gaba

Principle. GSH is reacted with PCMPS† at neutral pH to form a stable complex. Glu and GSH–PCMPS are adsorbed on an alumina column. Gline and Gaba present in the eluate are separated and purified by adsorption and elution from Dowex-50.

The alumina column. Alumina‡ is treated successively with twice the volume of 1 N-acetic acid, 1 N-NH_4OH and 1 N-HCl. After each treatment it is repeatedly washed with water by decantation until the wash water is neutral. This procedure serves also to remove the finer particles. The alumina is then stored under water.

* The following abbreviations are used in this paper: glutamic acid, Glu; glutamine, Gline; glutathione GSH; γ-aminobutyric acid, Gaba; trichloroacetic acid, TCA; *p*-chloromercuriphenyl sulphonic acid, PCMPS.

† Sigma.

‡ Reagent aluminum oxide (Merck); suitable for chromatographic adsorption.

The adsorption funnel is essentially as described before (PRESCOTT and WAELSCH, 1946). The column is formed by filling the funnel with water and introducing the suspension of alumina in water with a long-tip pipette. The column is packed by gently stirring the alumina with a fine wire and then flicking the glass tubing with the fingers or tapping with a pencil until the alumina does not settle further. The size of the alumina column is 2 × 0·3 cm.

The Dowex column. Dowex 50-x4–400 mesh is first converted to the sodium form according to MOORE and STEIN (1951). It is then transformed back to the hydrogen form (HIRS, MOORE and STEIN, 1952) and stored under water. For preparation of the columns, the resin is equilibrated with the buffer (ammonium formate 0·2 M, pH 3·1) (HIRS *et al.*, 1952), and stored under an equal volume of buffer. The resin column used is 12 × 0·7 cm and is formed by pouring the resin suspension into a glass tubing* with a tapered tip (opening 2 mm) and closed with a small plug of glass wool. The resin is allowed to settle by gravity, the final height adjusted to 12 cm and the column washed with 5 ml of the ammonium formate buffer.

Extraction of tissue, removal of GSH and Glu. Brain tissue (100–200 mg) is extracted four times with 1·0 ml of cold 5% TCA and the suspension centrifuged at 15,000 rev/min (International Refrigerated Centrifuge, high speed attachment). For larger amounts of tissue the volume of TCA is proportionately increased.

The combined extracts (4 ml) are placed into a graduated test tube and neutralized with 2·0 N-NaOH against indicator paper. A solution of 1·6% PCMPS in water (0·5 ml) is added and the volume made up to 6·2 ml with H_2O. The pH is checked with a pH meter and brought to 7·0 ± 0·1 with less than 0·01 ml of 1 N-acid or base.

Two 3 ml aliquots (for duplicate determinations) of the neutralized extract are passed through alumina columns. The columns are washed twice with 1 ml of water and the washings combined with the effluent. Glu and the GSH-PCMPS complex remain on the column while Gline and Gaba are not adsorbed and are quantitatively recovered in the eluate. Glu is eluted from the alumina column with three portions of 5 ml of 0·5 N-acetic acid. Subsequently, GSH is removed from the column with two 2 ml portions of 0·5 N-NH_4OH.

Separation of Gline and Gaba. The eluate of the alumina column (5 ml) is brought to pH 2·0 by the addition of 0·25 ml of 0·2 N-HCl (pH meter). A 5 ml aliquot is passed through the Dowex column and the sides of the column washed down with two 0·5 ml portions of 0·01 N-HCl. The column is eluted with 12 ml of 0·2 M-ammonium formate buffer pH 3·1 (HIRS *et al.*, 1952). The 5–6, 6–8 and 8–9 ml fractions are separately collected and used for Gline determination. The amide is concentrated usually in the 6–8 ml fraction. The column is subsequently eluted with 10 ml of 0·2 M-ammonium acetate buffer, pH 5·0 (HIRS *et al.*, 1952). Fractions 4–6, 6–8 and 8–10 ml are collected separately. Gaba is concentrated usually in the 6–8 ml fraction.

Different lots of Dowex 50-x4 vary in their physical and chromatographic properties, as has also been observed by others (BOYD and SOLDANO, 1953; MOORE and STEIN, 1954). MOORE and STEIN (1954) adjust the Dowex 50-x4 by adding Dowex 50 of slightly different cross linkage. The length of the column may also be

* The glass tubing is carefully selected for uniformity; outer diameter, 9 mm, inner diameter, 7 mm.

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adjusted and different batches of Dowex 50 should be tested to find one giving the best performance.

Determination. The solutions of the isolated compounds may require concentration for analysis. This is achieved in graduated centrifuge tubes in a water bath at 60° with the aid of a gentle stream of filtered air. The volumes are concentrated to 0·5 ml.

It was found convenient to determine Glu in 2 ml aliquots, GSH in 2–4 ml aliquots, Gline in 0·5 ml aliquots (concentration thus not required) and Gaba in the entire 2 ml of eluate.

The final volume of 0·5 ml should contain 0·05–0·5 μmoles of the compound to be determined. To the GSH solution is added 0·035 ml of glacial acetic acid. Since Glu and Gline samples are already acidic, addition of glacial acetic acid is not required. Sodium nitrite solution, 0·05 ml (80 g in 100 ml of H_2O) is added. Pipettes with drawn-out tips are used. The mixture is allowed to stand at room temperature for 20 min and then 0·25 ml of 4 M-NH_2OH·HCl is added. After 5 min 1 ml of 1·3 N-NaOH is added followed after 10 min by 0·2 ml 9 N-HCl and 1 ml of 20% $FeCl_3$·6 H_2O in 1 N-HCl. For the determination of GSH it is necessary at this point to centrifuge for 10 min (2000 rev/min) prior to reading the colour density. PCMPS tends to precipitate out and may result in high values unless removed. Alumina dissolved from the column by ammonia redissolves in the hydrochloric acid.

The determination of Gaba requires modification because of the presence of the acetate buffer. The eluate (2 ml) is concentrated to 0·5 ml at 60° with the aid of a filtered stream of air. It is then allowed to react with nitrous acid for 2½ hr at room temperature. Hydroxylamine reagent (0·25 ml) is added, followed in 5 min by 1 ml of 1·7 N-NaOH. After 10 min the solution is made acidic with 0·25 ml of 9 N-HCl and 1 ml of the ferric chloride reagent added.

The developed colour is read at 540 mμ in a Beckman Spectrophotometer with a cuvette carrier modified for use with cells with a 5 cm light path (5 × 1 cm cells (Aminco)). The usual carriage is replaced by one suitable for macrocells (Beckman) with a special adapter tooled to hold the cells. The cells require a volume of approximately 3 ml.

A complete reagent blank (including passage through the columns) is run along with each series of determinations and subtracted from the sample readings. All samples are read against a colour blank of 1 ml ferric chloride reagent plus 2 ml water. Gas bubbles which may form in the solution and result in high readings can be eliminated by avoiding any delay in reading and by pouring the solution back and forth between the cell and a test tube.

Glu, GSH and γ-butyrolactone give the same molar extinction $E_{540}^{1\,cm} = 832$. The molar extinction of Gline is 95 per cent, while Gaba gives 60 per cent of the colour developed by Glu under conditions used for the determination of the latter. When Gaba is reacted with nitrous acid, it is partially converted to γ-butyrolactone. The γ-butyrolactone is in equilibrium with γ-hydroxybutyric acid (Long, McDevit and Dunkle, 1951). In the presence of concentrated ammonium acetate buffer used for the elution of Gaba, deamination is slowed but the equilibrium altered in favour of the lactone. In 20 min there is 46 per cent, in 1 hr 66 per cent, in 2 hr 73 per cent and in 18 hr 78 per cent conversion of Gaba to the butyrolactone.

Calculations. Based on the proportion of solution taken as described for brain

tissue the concentration of the four compounds in μmoles/g of tissue would be calculated as follows:

Glutamic acid $\quad \dfrac{1000}{\text{mg tissue}} \times \dfrac{6{\cdot}2}{3} \times \dfrac{15}{2} \times \text{reading} \times 0{\cdot}73$

Glutamine $\quad \dfrac{1000}{\text{mg tissue}} \times \dfrac{6{\cdot}2}{3} \times \dfrac{5{\cdot}25}{5} \times \dfrac{2}{0{\cdot}5} \times \text{reading} \times 0{\cdot}73 \times \dfrac{100}{95}$

Glutathione $\quad \dfrac{1000}{\text{mg tissue}} \times \dfrac{6{\cdot}2}{3} \times \text{reading} \times 0{\cdot}73$

(if the whole 4 ml are used)

γ-Aminobutyric acid $\quad \dfrac{1000}{\text{mg tissue}} \times \dfrac{6{\cdot}2}{3} \times \dfrac{5{\cdot}25}{5} \times \dfrac{0{\cdot}73 \times 100}{73} \times \text{reading}$

RESULTS

Many substances give varying intensities of colour with this method (Table 1) usually far below the colour intensity developed by equivalent amounts of Glu. Yet if present in sufficient concentration, they could result in high values. The above procedures of separation and determination were so devised as to reduce the possible interference of contaminating materials to negligible quantities.

Aspartic acid is retained by the alumina and partially eluted in the 0·5 N-acetic acid. It has 14 per cent chromogenicity as compared with Glu but its final concentration makes the interference small. If aspartic acid is present in higher concentrations 90 per cent of its colour-forming ability can be destroyed by placing the sample in a water bath at 50° for 1 hr, after addition of the glacial acetic acid and $NaNO_2$ reagent. Aspartic acid probably forms a β-lactone which would be much less stable than the γ-lactone formed by glutamic acid.

Under the conditions of the determination, the contamination of Gline and Gaba with other amino acids is negligible. When separated within several hours at room temperature, no loss of Gline is incurred despite its instability.

TALLAN, *et al.* (1954) reported a fairly complete analysis of cat brain extract. A synthetic brain extract was prepared according to their analysis, and to this was added 100 mg/100 ml of glucose as well as the expected quantity of TCA. Thus, simulating conditions to be encountered when extracting 100–200 mg of tissue, mixtures of Glu, Gline, Gaba and GSH were determined (Table 2). Each determination carried out on 3 ml of synthetic extract (equivalent to 100 mg of tissue) was done in duplicate.

The distribution of the four compounds in various areas of the adult rat brain was then determined. Rats were decapitated, the brains quickly removed and the dissected tissues immediately placed in dry ice. The tissues were weighed frozen and extracted as previously described. The TCA extracts were kept frozen until analysed. The results are presented in Table 3 and include four series of determinations each on four or five animals, except for the corpus callosum which includes two series of determinations, one on seven animals and the other on eleven animals. The cortex represents all the lobes.

The medulla and pons were consistently lower in Glu, Gline and GSH than the cortex. The sections of rhinencephalon removed and analysed were the olfactory bulbs and the olfactory tracts with their surrounding cortex. In each series the Gaba content

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TABLE 1.—COLOUR FORMATION OF AMINO ACIDS AND OTHER COMPOUNDS IN THE HYDROXAMIC ACID PROCEDURE

Compound	$E^{1\ mg}_{5\ cm}$ 3·035 ml	$E^{1\ \mu mole}_{5\ cm}$ 3·035 ml
	$\lambda = 540$	
Glutamic acid	9·31	1·370
Glutamine	8·90	1·300
γ-Aminobutyric acid	7·98	0·822
Glutathione	4·46	1·370
L-Alanine	0·20	0·018
β-Alanine	2·32	0·206
α-Aminoadipic acid	3·70	0·596
α-Amino*iso*butyric acid	0·50	0·052
Δ-Amino-*n*-valeric acid·HCl	1·42	0·217
Aspartic acid	1·28	0·170
Asparagine monohydrate	0·20	0·030
Arginine HCl	0·00	0·000
Cysteine HCl	0·20	0·031
Betaine	0·26	0·030
Cystine	0·26	0·062
Glycine	0·60	0·045
Histidine HCl·H_2O	0·32	0·067
L-Leucine	0·36	0·047
Hydroxyproline	0·00	0·000
L-Lysine HCl	0·52	0·076
DL-*iso*Leucine	0·64	0·084
Norleucine	0·44	0·058
L-Methionine	0·66	0·098
Sarcosine·HCl	0·00	0·000
Ornithine HCl	1·00	0·168
L-Proline	0·48	0·055
L-Phenylalanine	0·30	0·050
L-Serine	0·76	0·080
L-Threonine	0·70	0·083
L-Tryptophane	0·48	0·098
L-Tyrosine	0·46	0·083
L-Valine	0·74	0·087
DL-α-Amino-*n*-valeric acid (norvaline)	0·12	0·014
Citrulline	0·00	0·000
Taurine	0·23	0·029
Creatine	0·00	0·000
Ethanolamine	0·85	0·052
o-Phosphoethanolanine	0·31	0·044
Glucose	0·60	0·108
Lactic acid	1·88	0·169
γ-Butyrolactone	15·91	1·370

Each sample contained 50 to 100 μg per 0·5 ml of water. Acetic acid and nitrite were added as in the determination of GSH (see text).

TABLE 2.—THE RECOVERY OF GLUTAMIC ACID, GLUTAMINE, γ-AMINOBUTYRIC ACID AND GLUTATHIONE FROM 'SYNTHETIC BRAIN EXTRACT'*
(Duplicate aliquots of 3 ml each were analysed)

	Found μmoles	Added μmoles	Found μmoles	Added μmoles	Found μmoles	Added μmoles	Found μmoles	Added μmoles	Found μmoles	Added μmoles	Found μmoles	Added μmoles
Glutamic acid	0·95		0·50		0·98		0·56		0·97		0·47	
	1·01	1·0	0·49	0·50	0·96	1·0	0·48	0·50	0·98	1·0	0·48	0·50
Glutamine	0·49		0·23		0·45		0·25		0·47		0·44	
	0·50	0·50	0·25	0·25	0·49	0·50	0·25	0·25	0·47	0·50	0·44	0·48
γ-Aminobutyric acid	0·25		0·121	0·125	—	—	—	—	—		0·22	
	0·28	0·25	0·130						0·25	0·25	0·24	0·25
Glutathione	0·22		0·11		0·21		0·12		0·21		0·20	
	0·22	0·20	0·11	0·10	0·21	0·20	0·11	0·10	0·21	0·20	0·20	0·20

Soln. A—Neutral amino acids (mg/100 ml)		Soln. B—Basic amino acids (mg/100 ml)		Soln. C (mg/100 ml)	
Asparagine	1·4	Lysine HCl	2·4	Aspartic acid	30
Glycine	10·1	Histidine HCl	1·1	Taurine	24
Alanine	8·4	Arginine	1·6	Creatinine	50
β-Alanine	0·6			Urea	25
Valine	2·1			Ethanolamine	21
Leucine	1·8			Phosphoethanolamine	42
*iso*Leucine	1·2			Glucose	100
Serine	7·6				
Threonine	2·6				
Methionine	1·5				
Cystine	1·0				
Proline	1·6				
Phenylalanine	1·2				
Tyrosine	1·2				

* Synthetic brain extract = 0·2 ml solutions A, B and C 4 ml 5% TCA 0·2–0·4 ml of these solutions containing Glu, Gline, GSH and Gaba.
Final vol. = 6·2 ml equal to 200 mg of brain.

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TABLE 3.—GLUTAMIC ACID, GLUTAMINE, γ-AMINOBUTYRIC ACID AND GLUTATHIONE IN ADULT RAT BRAIN
(μmoles/g tissue*)

	Cortex	Thalamus and hypothalamus	Medulla	Pons	Cerebellum	Olfactory bulbs and tracts	Corpus callosum	Remainder of brain
Glutamic acid	10·4–12·5	9·5–12·5	5·1–5·9	6·3–6·7	10·0–10·4	9·1–10·5	8·6†–9·3‡	9·1–10·6
Average	11·6	10·7	5·7	6·4	10·2	9·8		9·8
Glutamine	4·6–5·4	3·0–6·0	2·3–4·1	2·9–3·8	5·3–6·4	4·0–6·3	3·8†–4·6‡	4·5–5·8
Average	5·0	4·8	3·0	3·2	5·8	5·1		5·1
γ-Aminobutyric acid	1·5–2·7	2·4–5·2	0·8–3·0	1·5–2·1	1·1–2·1	2·2–4·6	0·5†–0·6‡	2·0–3·4
Average	2·0	3·5	1·5	1·7	1·6	3·2		2·6
Glutathione	2·1–2·5	1·9–2·5	1·0–1·6	1·2–1·5	1·7–2·2	1·8–2·4	2·1‡–2·2†	1·6–2·3
Average	2·3	2·1	1·2	1·4	1·9	2·0		1·9

* Four series of determinations in duplicate, each series on the combined tissues from four to five animals.
† Combined tissues from eleven animals.
‡ Combined tissues from seven animals.

was higher than in the cortex. The corpus callosum had a considerably lower concentration of Gaba than any other part of the brain. For analytical data reported by others, a recent review may be consulted (WAELSCH, 1957).

In our experience over the last two years, five to six separate tissue samples can be analysed for Glu, Gline, Gaba and GSH within 4 to 5 days by one person.

Sensitivity of the method. Glu, 0·1 μmole, will give a reading of 0·137 $\pm$ 0·005 at 540 mμ in a 5 cm cell. Approximately the same deviation is found at lower concentrations. Therefore, amounts down to 0·01 μmole of Glu are detectable with a corresponding decrease in accuracy. The same situation holds true for Gline and GSH, while higher concentrations of Gaba are needed since the molar extinction of this compound in the determination corresponds to 73 per cent of that of Glu.

While this degree of accuracy applies to the determination of Glu, Gline and Gaba, the error in the GSH determination may sometimes be greater owing to the fact that any Glu retained in the alumina column is eluted by the ammonia solution and would then be determined as GSH. Such an error will occur particularly in tissue such as brain where the ratio of concentration of Glu to GSH is approximately 5 : 1 but the interference will be less in tissues such as the liver where the ratio is about 1 : 2.

For determination of Glu and its metabolic derivatives in tissues other than the brain, amounts of tissue taken have to be adjusted according to concentrations to be expected. For instance, in several hundred determinations of Glu and Gline in human blood serum by this method, 1 ml of serum was employed and the PCMPS treatment as well as adsorption on Dowex omitted. The Glu was adsorbed on the alumina column. The Gline in the eluate was hydrolysed to Glu (SCHWERIN, BESSMAN and WAELSCH, 1950) which was adsorbed on and eluted from an alumina column and determined as described above.

The method as outlined lends itself not only to the determination of Glu, Gline, Gaba and GSH but is also useful in metabolic studies with isotopes since the separation of the various compounds is sharp enough as to eliminate significant cross contamination. (See LAJTHA *et al.*, 1959).

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SUMMARY

(1) A micromethod is described for the determination of glutamic acid, glutamine, γ-aminobutyric acid and glutathione in 100–200 mg of brain tissue or corresponding amounts of other tissues or body fluids.

(2) Data on the concentration of glutamic acid, glutamine, γ-aminobutyric acid and glutathione in different areas of the adult rat brain are presented.

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CHANGES IN THE COMPOSITION OF THE DEVELOPING MOUSE BRAIN DURING EARLY MYELINATION*

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RECENT interest in changes in the composition of the mammalian brain during the early maturation stage undoubtedly results from the recognition that the study of such chemical changes, when combined with a study of morphogenesis, may suggest the localization (or function) of chemical components of brain. On the other hand, the relatively static compositional and histological picture of the adult brain offers no such promise.

Earlier efforts were hampered by the inadequacy of analytical methods available (KOCH, 1904; KOCH and CARR, 1909; KOCH and KOCH, 1913; DONALDSON, 1916; MACARTHUR and DOISY, 1919) and by the fact that the chemical development of brain was studied only with respect to a few constituents (DONALDSON, 1916; MAC ARTHUR and DOISY, 1919). The paucity of parameters and the lack of a significant frame of reference rendered interpretation of such results difficult. The greatest amount of work done in this respect has naturally focused on the changes in the patterns of brain lipids because a superficial parallelism between increase in lipid content, increase of myelination, and general maturation of brain function, in terms of motor co-ordination and behavioural patterns, was evident. This parallelism appeared to justify intensive work on changes of known brain lipids. This approach, though sporadically taken up during the past 30 years, suffered not only from analytical shortcomings, but also from lack of information as to what all the lipid constituents of brain were. Perhaps the most significant information on the rapidity with which compositional changes occur in the developing brain was provided by the early studies of WAELSCH *et al.* (1941) which showed the high rate of incorporation of labelled fatty acids into myelin lipids during early myelination. The efforts to date have recently been critically reviewed (SPERRY, 1955; FOLCH-PI, 1955; LEBARON and FOLCH, 1956). More recent work (FOLCH-PI, 1955; SPERRY, 1955) has investigated the nature of the changes in the lipids of the developing brain, and the information thus gained has superseded the extensive survey of BRANTE published in 1949. It is thus generally conceded that the maximal content of cerebrosides and sphingomyelins corresponds to that phase in the development of mammalian brain in which histologically detectable myelination reaches its maximum level (KOCH and KOCH, 1913; FOLCH-PI, 1955; BRANTE, 1949), and that the appearance of gangliosides (strandin) and phosphatides antedates this phase (FOLCH-PI, 1955). The major unproven assumptions in these tentative conclusions are (a) that increase in a lipid component

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represents myelin–lipid, because it appears to increase *pari passu* with the increase in myelin, (b) that the number of cells and cell types in brain remains constant during maturation so that the first assumption becomes possible, and (c) that the lipid components within various neuronal and glial elements remain qualitatively and quantitatively constant. It is with these reservations, therefore, that all interpretations of lipid distribution within the developing brain have to be made, especially when the lack of homogeneity of the immature brain in morphological constituents, and the absence of any clear distinction between 'grey' and 'white' matter, are taken into account.

The present study was originally undertaken with the purpose of investigating the relationship of water-soluble glycolipids to the process of myelination in the immature mouse brain. In 1953 we had suggested the possibility that water-soluble glycolipids of the polycerebroside class might represent the precursors of cerebrosides, in as much as partial hydrolysis of polycerebrosides *in vitro* yielded a substance indistinguishable from kerasin (UZMAN, 1953*a*). This view was also later expressed by KLENK (1955) with regard to the role of gangliosides and polycerebrosides. In the preliminary phases of the present study it became evident, however, that changes in the water-soluble glycolipid constituents of the immature brain had to be studied within a broader frame work defined not only in concomitant changes in other brain lipids, but also with respect to changes in total nuclear number, total cytoplasmic mass (including cell processes), and other non-lipid constituents. Towards this end, changes in total nucleic acids and total non-lipid residue content taking place with progressing maturation of the brain were determined, and changes in desoxypentose nucleic acids and ribose nucleic acids were used as indices for changes in total nuclear number and total metabolically active cytoplasmic mass, respectively.

The results of this study indicate that the bulk of the 'myelin-lipids' are derived from existing lipids in the developing nervous system, cerebrosides alone being added to the composition of the brain as myelination and maturation progresses. It is further observed that water-soluble glycolipids undergo significant compositional changes both before and after cerebrosides make their appearance. These changes are interpreted as indicative of their probable role as precursors to cerebrosides.

MATERIALS AND METHODS

The brains of Wistar strain Swiss albino mice bred at the New Animal House, Harvard Medical School, were used in this study. Animals belonging to the 1, 3, 5, 7, 10, 12, 14, 16, 18, 20, 23, 42 day and adult (10 week) age groups were sacrificed by section of the spinal column immediately below the foramen magnum. Cerebrum, cerebellum and pons were removed in one piece within 1 min after death, immediately weighed in batches of 5–10 in weighing bottles and frozen (2–24 hr) until processed. The number of animals used (Fig. 2) varied according to the size of the brain at different ages, as many as sixty-five animals being used for the 1 day group, and twenty for the adult. Fewer animals were used in the 16, 18 and 23 day groups as these were added to the series to confirm the important changes discovered to occur between 14 and 20 days.

Histological controls were done on the 5 and 7 day group by the copper-impregnation stain (UZMAN, in preparation), while serial sections of the 10, 14, 20 day and adult animals were stained with Nissl and Luxol Fast Blue (KLÜVER and BARRERA, 1953). Non-serial sections from all age groups were examined with osmic acid fixation staining, with PAS staining after Rossman fixation (HESS, 1953), and with the copper-impregnation method after formalin fixation.

The following scheme was used for analysis. The frozen mouse brains (from ten to twenty at a time, or 2–6 g fresh tissue) were allowed to thaw at room temperature and homogenized with 20 ml/g tissue with chloroform–methanol (2 : 1 v/v). The homogenates were allowed to boil for 3 min in a

water-bath at 70°C and were filtered into 250 ml volumetric flasks. In our experience, the use of boiling solvent for this short period was found necessary for maximum recovery of lipids. The residue was extracted twice more with boiling chloroform–methanol, and the filtrates combined with the first. The combined filtrates were made up to volume with chloroform–methanol. The brains were thus divided into a chloroform–methanol soluble fraction (total lipid extract), and a chloroform-methanol insoluble residue (total residue). The latter was air-dried to constant weight and the weight recorded.

The values for total lipid content of the brains were obtained by taking aliquots of the total lipid extract to dryness in pre-weighed evaporating dishes, first in a stream of air, then in high-vacuum until constant weight was attained. The reason the 'washed' lipid extract was not used for this purpose (FOLCH *et al.*, 1951) was that the difference was found to amount to less than 4 per cent when allowance

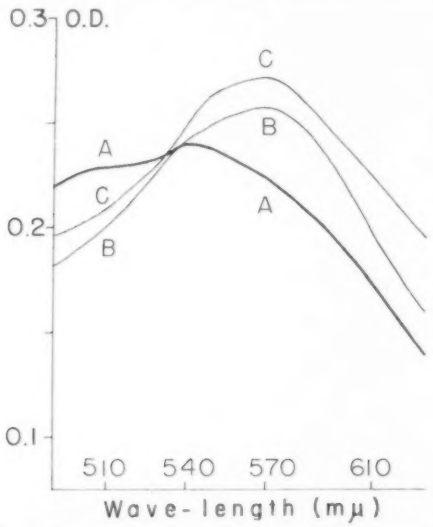

FIG. 1.—Absorption spectra of reaction products with Bial's reagent. The absorption maximum with mouse brain lipid extracts (A) is at 540 mμ, while that given by serum protein-bound neuraminic acid (C) and purified crystalline methoxy-neuraminic acid (B) is at 570 mμ.

was made for water-soluble glycolipids present, and because the latter were considered truly a part of the 'lipid' content of the organs.

Total lipid phosphorus was determined in aliquots of the total lipid extract (SPERRY, 1942) and considered to represent total phosphatide phosphorus. This was justified by the finding that about 96 per cent of the total phosphorus in the chloroform–methanol extract, after partitioning in a water–chloroform system as described below, was recovered in the chloroform phase. Since no attempt was made to subfractionate the phosphatides, the conventional calculation of total phospholipid from lipid–phosphorus figures by multiplying the latter by the factor 25 was omitted, the lipid phosphorus itself being presented.

Neuraminic acid was determined according to BÖHM *et al.* (1954) in aliquots of the total lipid extracts. Since there is no unanimity as to the neuraminic acid content of gangliosides (KLENK, 1955), the data for neuraminic acid are presented as an indirect measure of ganglioside content. It should be pointed out that both the procedure of BÖHM *et al.* (1954), as well as that of KLENK and LANGERBEINS (1941), lack the desired specificity, the coloured reaction products of sugars interfering with the development of the correct colour, and the determination of gangliosides suffers from this handicap. Our data and interpretations based on neuraminic acid content should be considered with this reservation in mind. The reaction product of aliquots of brain lipids with Bial's reagent had an absorption maximum at 540 mμ while that of serum neuraminic acid and purified methoxy-neuraminic acid was at 570 mμ (Fig. 1).

The determination of total water-soluble glycolipids (gangliosides plus polycerebrosides) was

effected by the following process, derived from our earlier observations indicating that chloroform–methanol extracts of tissue will give clear-cut separations when partitioned against an aqueous phase of high dielectric constant, allowing the recovery of water-soluble glycolipids in the aqueous phase (UZMAN, 1953*a*) and retaining cerebrosides in the organic solvent phase (UZMAN, 1953*b*). It should be emphasized that the total water-soluble glycolipids as measured here include the ganglioside strandin described and studied by FOLCH-PI (1955). Aliquots of the total lipid extract (10–20 ml) were taken to dryness, with the addition of a few granules of 60-mesh alundum, in glass-stoppered borosilicate 15 ml centrifuge tubes in a water-bath at 70°C. To the lipid residue 4 ml of water and 0·1 ml of a 10% solution of sodium chloride were added. The tubes were stoppered and shaken thoroughly until the lipid formed a white, homogenous emulsion. To each tube 2·0 ml of chloroform was next added, and the tubes were intermittently shaken and then allowed to stand at room temperature overnight. They were then centrifuged for 1 hr at 2500 G. Three layers were obtained: a clear aqueous top layer, a well-packed interphase layer, and a bottom chloroform layer. Aliquots of the top layer were carefully pipetted off, and the hexose content determined with orcinol–sulphuric acid according to a modification of the method of BRÜCKNER (1942). The results were expressed as glucose equivalents. Other aliquots were used for the determination of the total reducing value before hydrolysis, according to an adaptation of the reductimetric procedure of FOLIN and MALMROS (1929). Still other aliquots were removed, concentrated *in vacuo*, and used for the chromatographic determination of free sugars using the pyridine–ethyl acetate–water system of MONTREUIL *et al.* (1953). No free glucose or galactose could be detected chromatographically (MONTREUIL *et al.*, 1953) in the total lipid extracts, hence no correction was made for non-lipid hexose in the total lipid extracts. That the partition procedure used was effective was evidenced by the following: (a) reproducibility of total water-soluble hexose was uniformly better than 2 per cent, (b) over 95 per cent of the neuraminic acid content of the total lipid extract was recovered in the aqueous phase, (c) no glucose could be demonstrated chromatographically after hydrolysis of the chloroform phase, the sole sugar being galactose, and (d) 96–102 per cent of pure cattle-brain cerebroside, added to the extract before partition, was recovered in the chloroform phase.

The determination of total lipid hexose was carried out in two steps. In the first, aliquots of total lipid extract were taken to dryness in a water-bath at 70°C. To the dry lipid residue 2·0 ml of N-HCl was then added and the sugars liberated by hydrolysing for 2 hr in a boiling water-bath, in a modification of the procedure of MONTREUIL *et al.* (1953). The exact conditions for this hydrolysis were determined by studying variations in heating-time, temperature and acid concentration on minimum destruction of the sugars. The conditions finally used gave 96–102 per cent recovery for the pure sugars, and 98 per cent recovery for hexose added in the form of cerebrosides. The hydrolysed samples were cooled, and the water-soluble portion quantitatively transferred, with distilled water washings, to a 5 ml volumetric flask and made up to volume. The heavy lipid flocculate was allowed to settle out as a coarse precipitate by keeping the hydrolysates (5 ml) in the refrigerator overnight. The hydrolysates were then filtered through micro-sintered glass filters under minimum negative pressure. In the second step of the procedure, aliquots of the water-clear filtrates were used to determine hexose content with the orcinol–sulphuric acid procedure of BRÜCKNER (1942). Results were expressed as glucose equivalents. In all cases samples were hydrolysed in duplicate, and aliquots thereof also analysed in duplicate. From the total lipid hexose values (expressed as glucose equivalents) the water-soluble glycolipid hexose (as glucose equivalents) was subtracted, yielding figures for cerebroside–hexose (as glucose equivalents). The latter was converted to galactose equivalents by multiplying with the factor 0·74 which was determined experimentally from the colour values of standard glucose and galactose curves obtained by subjecting different concentrations of the two sugars through the whole procedure.

The total residue, representing the moiety of brain tissue insoluble in boiling chloroform-methanol, was weighed after thorough drying to constant weight, as previously described. Samples of each age group were submitted to analysis for phosphorus (SPERRY, 1942). Other aliquots were extracted with hot trichloroacetic acid according to SCHNEIDER (1945), and the phosphorus content of the trichloroacetic extracts compared with the total nucleic acid content determined by other procedures. Other aliquots of the total residue were extracted with 10 per cent perchloric acid according to CERIOTTI (1955). The absorption of these extracts at 262 mμ (Beckman D.U. Spectrophotometer), compared with that of a highly purified and thoroughly characterized preparation of deoxyribonucleic acid (DNA) from normal human spleen (UZMAN and DESOER, 1954) was used to calculate total nucleic acid

concentration, independently from that based on the SCHNEIDER procedure (SCHNEIDER, 1945). The residues were analysed with orcinol, according to CERIOTTI, for the total ribose nucleic acid content (RNA) (CERIOTTI, 1955). The RNA content was calculated from the ribose figures obtained analytically by multiplying by the factor 2·17 (the theoretical ribose content of RNA). Deoxy ribonucleic acids were calculated as the difference between total nucleic acid and RNA.

Cholesterol was not studied in as much as the changes occurring in the developing brain are already adequately documented in the literature (FOLCH-PI, 1955; SPERRY, 1955; BRANTE, 1949).

RESULTS

Changes in overall composition

In the particular strain of mice studied, the average weight of the brain showed a steady sharp increase up to the sixteenth day, but from then on a steady decline of average brain fresh weight was recorded until the twenty-third day. This trend was reversed after the twenty-third day, and the brain weight rapidly rose above the 16

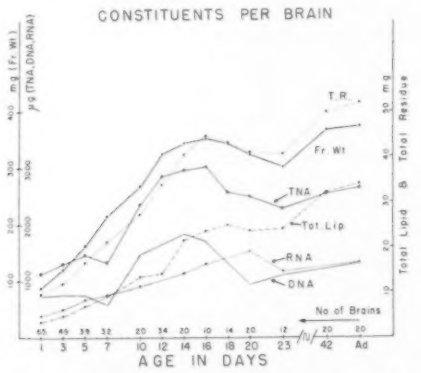

FIG. 2.—Changes in the absolute amounts of fresh weight (Fr. Wt.), total lipid-free residue (T.R.), total lipid (Tot. Lip.), total nucleic acid (TNA), total DNA and RNA in the course of maturation of the mouse brain.

day maximum by the forty-second day, showing little difference between then and the 10 week adult mouse brain. The changes in brain weight (with number of animals from which averages were calculated) as well as the content of each constituent, expressed as per brain are presented in Fig. 2. In spite of this loss in fresh weight after the sixteenth day, the total dry weight (calculated as total residue plus total lipid) showed a steady increase from the 1 day brain to the adult (Table 1). This difference reflects rapid loss of water of the mouse brain after 16 days. The lipid-free residue of brain (total residue) showed a steady increase until the fourteenth day, a small decline from 16–23 days, and a small increase from then on to the adult level. At 23 days the total residue (including total protein and nucleic acids) had reached 81 per cent of the content of adult brain. Total nucleic acid (TNA) reflected the changes in total brain weight, showing a maximum level at 16 days, a steady decline to 23 days, and from then on a steady increase to the adult. However, the total nucleic acid content of the 16 day brain was still about 12 per cent higher than that of the adult. This fluctuation of the total nucleic acid content of brain was found to be essentially a reflection of the changes in its total DNA content. The latter reached a peak level at about 14 days, dropped off sharply by 23 days (losing about 25 per cent) so that the adult brain DNA

content was about 10 per cent below the 14 day animal brain. On the other hand, the RNA content showed a progressive rise with age, reaching a maximum by 20 days, and showing similarly a 10 per cent reduction from that level in the adult animal. Thus, the decrease in DNA content (starting around 14 days) antedated the decrease in brain weight which became evident after 16–18 days. The drop in DNA was reflected by a drop in RNA also after the twentieth day.

The small decrease in brain weight recorded after 16 days was re-examined at the suggestion of the referees almost a year after the completion of the whole study. Eight litters of the same strain of mice originally used (with at least eight mice per litter) were examined for the variations in brain weight between the fourteenth and thirtieth day of life. Individuals from each litter were sacrificed at appropriate intervals by severing the spinal cord at the level of the foramen magnum. The

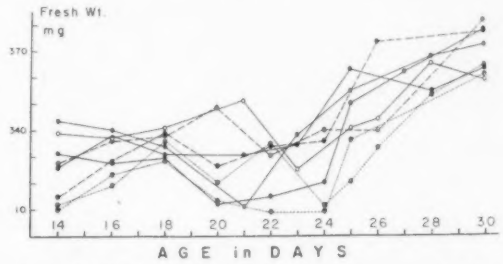

FIG. 3.—Showing the individual brain fresh weight growth curves of eight litters during the 14–30 day period.

individual brains were removed rapidly and weighed in glass-stoppered weighing bottles. Thus, the brain-weight growth curve was obtained for each litter. The results are given in Fig. 3. None of the litters showed a steady increase in brain weight between 18–24 days, the majority showed a distinct loss. In some this decrease extended over more than a single measured interval. In two instances the interval in question 18–24 days was represented by a plateau curve. The decrease was not of the same magnitude nor did it occur at the same time in different litters. However, the dating of each litter was only possible for a given 24 hr period so that litters 1 hr old would be classified as being of the same age as those 24 hr old. Thus, some of the differences may be due to this factor and some to the unavoidable sampling error, so that days on which maximum weight changes occurred were missed. The period of decrease in brain weight contrasted strikingly with the 26–30 day period where there was a marked gain in brain weight in all litters.

The total lipid content showed a progressive increase with age, showing a rapid but steady rise to the sixteenth day (almost a sevenfold increase over the 1 day animal), a period of relatively no increase between the 16–23 days, and a smaller rise to the adult stage. Thus, by 16 days the mouse had acquired 69 per cent of the lipid that it would possess as an adult, and by the twenty-third day about 70 per cent of the adult lipid content (8·5 per cent of fresh weight). The lipid phosphorus reflected the total lipid in this respect, showing a rapid and steady rise to 16 days (a 650 per cent increase over the 1 day animal) a constant level between 16 and 23 days and a rise from there on to the adult stage (Table 1). At 23 days the mouse brain contained about 75 per cent of the total lipid phosphorus of the adult brain. The total lipid hexose (reflecting both

TABLE 1.—PERCENTAGE COMPOSITION OF DEVELOPING MOUSE BRAIN

Age (days)	1	3	5	7	10	12	14	16	18	20	23	42	Ad
Average wt. of brain (mg)	88	120	162	219	266	324	343	351	343	322	301	367	372
Total lipid as % fresh wt.	3·87	4·00	4·17	4·49	5·06	5·96	6·45	6·72	7·02	7·36	7·70	8·70	8·50
Total residue as % fresh wt.	10·06	9·60	10·13	10·38	10·30	11·25	11·80	12·50	12·59	12·70	13·10	13·61	13·80
Total dry wt. (total lipid + total residue) as % fresh wt.	14·6	13·7	14·5	13·9	15·3	16·3	18·1	19·4	19·8	20·0	21·4	22·3	22·9
Lipid phosphorus as % total lipid	2·44	2·37	2·42	2·41	2·53	2·38	2·38	2·49	2·34	2·36	2·42	2·34	2·24
Neuraminic acid as % total lipid hexose (glucose equivalent)	26·0	25·4	22·6	20·6	16·95	25·4	12·35	18·4	14·01	10·25	15·7	9·45	9·35
Total lipid hexose (glucose equivalent) as % total lipid	1·28	1·41	1·58	1·83	2·21	2·68	2·56	2·34	2·62	3·11	2·5[illegible]	3·78	3·45
Cerebroside hexose (galactose equivalent) as % total lipid	0·00	0·00	0·00	0·00	0·71	0·99	1·41	1·00	1·28	1·60	1·2[illegible]	2·28	1·98
Water-soluble glycolipid hexose (glucose equivalent) as % total lipid hexose	100	100	100	100	56·8	51·5	40·1	41·4	32·6	32·3	34·5	18·45	22·7
Neuraminic acid as % W-S glycolipid hexose (glucose equivalent)	26·0	25·4	22·6	20·6	29·4	62·0	30·9	46·2	43·0	33·1	44·9	51·0	42·4
Reducing value (glucose equivalent) as % W-S glycolipid hexose	39·3	38·3	25·9	21·6	25·7	66·8	33·4	27·0	–	21·1	24·2	–	20·2
Total residue phosphorus as % total residue	1·28	1·41	1·17	1·07	1·32	–	1·17	1·14	–	1·10	0·98	–	0·88
Total nucleic acid as % total residue	11·86	10·80	8·65	6·40	8·67	8·38	7·36	6·79	6·00	6·14	5·63	5·14	4·65
Total residue phosphorus as % total nucleic acid	10·81	13·05	13·53	16·50	15·22	–	15·88	16·78	–	17·93	17·33	–	18·97

cerebroside hexose as well as water-soluble glycolipid hexose), showed a steady increase till the tenth day, a very steep rise between 10–14 days, almost doubling itself in that period (95 per cent), a rather even level until the twenty-third day, from which time on a rather steep rise was again observed until the adult stage was reached. The 23 day animal possessed only 53 per cent of its total lipid hexose as compared with the adult.

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Water-soluble glycolipids

The total lipid hexose reflected the compositional changes occurring in the water-soluble glycolipids and the cerebrosides. Until the seventh day, all the lipid hexose was in the form of water-soluble hexose (Table 1). From there on cerebrosides made their appearance, showing a sharp rise to 14 days and a more steady but constant rise, both in absolute amount (Fig. 4) as well as with respect to total lipid and total lipid hexose (Table 1), until the adult stage was reached. The water-soluble glycolipid, on

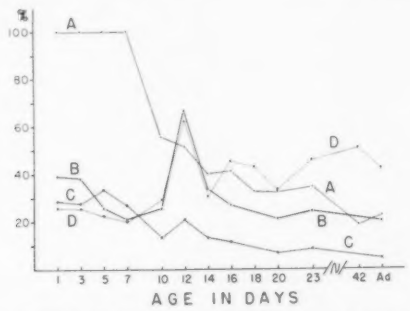

FIG. 4.—Variations in the composition of water-soluble glycolipid fractions of mouse brain at different ages. (A) represents water-soluble glycolipid hexose (gluc. equiv.) as a percentage of total lipid hexose; (B) neuraminic acid as a percentage of water-soluble glycolipid hexose; (C) reducing value of unhydrolysed total water-soluble glycolipid (gluc. equiv.) as a percentage of total lipid hexose (gluc. equiv.), and (D) shows the variation of the reducing value (gluc. equiv.) of the unhydrolysed water-soluble glycolipid fraction, expressed as a percentage of the water-soluble glycolipid hexose, at different ages.

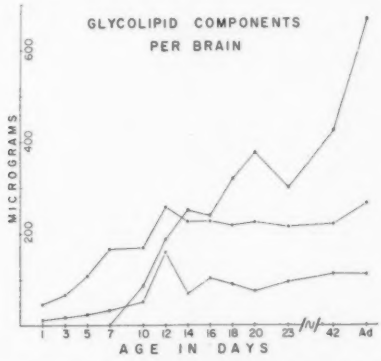

FIG. 5.—Changes in the content of glycolipids of brain during maturation. (○—○—○) represents cerebroside content as galactose equivalents per brain; (●—●—●) represents the total water-soluble glycolipid hexose content as glucose equivalents (representing gangliosides plus polycerebrosides); and (×—×—×) represents the neuraminic acid content.

the other hand, increased steadily until the twelfth day and from then on showed no significant increase until the adult stage (Fig. 5). It accounted for a constantly decreasing moiety of the total hexose, with a reduction from 100 per cent of the total lipid hexose at 5 days to 26 per cent in the adult (Fig. 4 and Table 1). When the total water-soluble hexose was compared with the total reducing value of the water-soluble glycolipids in the aqueous phase of the partition, a constant relationship was not observed, indicating that the glycolipids in this phase were not of identical nature

throughout all ages. Thus, the total reducing value (before hydrolysis) of water-soluble glycolipids as a percentage of total water-soluble hexose rose from 1 day on, reaching an abrupt peak at 12 days. The same sharp rise was observed when the experiment was repeated on two other separate occasions. From there on, there was a gradual decline in this relationship with wide fluctuations until the adult stage was reached (Fig. 4). The neuraminic acid in the lipid extract also showed a marked inconstancy with respect to the water-soluble glycolipids at different ages, so that, when expressed as a percentage of total water-soluble glycolipid, the ratio showed a constant increase from 1 day on, reached an abrupt and sharp peak at 12 days, and from then on fell precipitously between 14–16 days, subsequently showing again a gradual rise from the sixteenth day to the adult stage. These observations were confirmed by repeated analyses in two experiments. This undoubtedly indicates the presence of more than one type of water-soluble glycolipid in the aqueous phase after partitioning, and that the proportion of these water-soluble glycolipids changes with different ages, resulting in different mixtures of gangliosides (water-soluble glycolipid with neuraminic acid) and polycerebrosides (water-soluble glycolipid without neuraminic acid) to be present in different age groups (Fig. 4), thus accounting for the different 'water-soluble lipid hexose/reducing value before hydrolysis' and 'water-soluble lipid hexose/neuraminic acid' ratios at different ages.

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Nature of the phosphorus in the lipid-free residue

In all age groups a marked discrepancy between the phosphorus content of the total residue (lipid-free) and its TNA content was observed. The phosphorus not extracted by boiling chloroform–methanol was in excess of the amount that would be expected from the total nucleic acid content irrespective of whether the latter was extracted according to SCHNEIDER (1945) or CERIOTTI (1955). The difference became greater as the brains matured, so that the adult brain contained the greatest amount of non-nucleic acid phosphorus in its lipid-free residue (Table 1). In order to evaluate this further, a separate experiment was performed on 12, 18, 42 day and adult mouse brains. The phosphorous content of a hot 10 per cent perchloric acid extract (70°C for 20 min) of the respective total residues was compared with the total nucleic acid content of the same extract. The latter was determined by the extinction at 262 mμ of the extract (diluted appropriately with 10 per cent perchloric acid), as compared with a pure DNA standard preparation similarly treated. Table 2 shows the disparity between the phosphorus and the nucleic acid. This disparity increased with increasing

TABLE 2.—CHANGES WITH PROGRESSING MATURATION OF BRAIN IN THE P/TNA RATIO IN THE HOT 10% PERCHLORIC ACID EXTRACT OF THE TOTAL RESIDUE OF MOUSE BRAIN

Age (days)	12	18	42	Adult
P as % total residue	1·12	0·99	0·86	0·94
TNA as % total residue	8·16	5·97	5·13	4·27
P as % TNA	13·75	16·55	16·75	21·98

age. The phosphorus represented 13·75 per cent of the total nucleic acids in the 12 day brain (expected value should be 8–9 per cent) and 21·98 per cent of the total nucleic acid in the adult brain. This was taken to indicate the presence of one or more phosphorus-containing, non-nucleic acid, non-lipid fractions in the mouse brain. These fractions apparently increase steadily throughout the development and maturation of the brain presumably representing inositol phosphatides and phosphatidopeptides (LEBARON and FOLCH, 1956). Although extraction of the total residue with 10 per cent perchloric acid for 20 min at 70°C resulted in the extraction of all the phosphorus present in the lipid-free residue, extraction with 5% trichloracetic acid for 15 min at 90°C, according to SCHNEIDER (1945), gave recoveries between 90–93 per cent for most of the age groups. To test the completeness of the extraction of nucleic acids from the total residue, the total residue of brains of different ages were subjected to extraction with 10% perchloric acid at 70°C for 20 min. The precipitates were washed with 10% perchloric acid, centrifuged off, and then hydrolysed in sealed tubes with formic acid for 4–6 hr (UZMAN and DESOER, 1954). The formic acid was removed *in vacuo*, and the respective hydrolysates were chromatographed for the detection of purines and pyrimidines. None could be detected in this manner, indicating that the perchloric acid extraction of nucleic acids was quantitative. Hydrolysis of the total residue, after extraction of the nucleic acids with hot perchloric acid, with 6N-HCl in sealed tubes at 110°C for 6–8 hr, and subsequent chromatographic analysis, with *n*-butanol–acetic acid–ethanol–water (45 : 10 : 35 : 10 v/v) as the developing solvent, revealed traces of glucosamine, glucose and two other unidentified sugars as detected with ammoniacal silver nitrate. The amount of such reducing substances was estimated to account for less than 0·1 per cent of the total residue freed of nucleic acids by the perchloric acid extraction.

Histological examination

The purpose of this aspect of our study was not a systematic documentation of the order and degree of myelination of the developing mouse brain, but rather to assess the amount of myelinated fibres within those large masses of fibre tracts which, in the adult, contribute the largest sample of analytically detectable lipid sources. In the mouse, this is restricted to the calloso-tapetal complex which constitutes the main bulk of the myelinated structure, the anterior and posterior commissures, and the fronto-occipital association fibres. Hence, our studies of serial sections with a variety of myelin stains were not aimed at recording the appearance of isolated myelinated sheaths around a few fibres of a large tract but rather to record those substantial additions of myelinated fibres in the course of development that would be of analytical significance. Thus, although isolated myelinated fibres are quite evident in the pons, brachium conjunctivum, and the decussations of the brachium conjunctivum in the mid-brain by 10 days of age, the total amount of myelin in these structures even in wholly myelinated adult brain was estimated to represent a small fraction of the total myelinated mass analysed. Hence, for the purposes of the analyses carried out in this study, we feel it fair to state that up to 10 days of age the mouse brain was substantially free of myelin. Almost the whole hemispherical substance consisted of the cerebral mantle, and the 'fountains' of glial cells extending out from the whole lateral aspects of the lateral ventricles. Early myelin formation was quite evident in the pons, in the mesencephalic and diencephalic portion of the spino-thalamic tracts and the

interpeduncular commissures. The corpus callosum, the tapetum and the anterior commissure showed essentially no myelination. The extremely heavy cellularity of the cerebral mantle and the lateral walls of the lateral ventricles is illustrated in Fig. 6. The histological picture was similar at 14 days, the corpus callosum and tapetum still being largely devoid of myelin, while the cerebral mantle showed even higher cell density as the glia from the ventricular angles extended outwards throughout the hemispheres. At this stage mature and immature oligodendroglia were arranged in closely packed cordons or columns between and along almost all unmyelinated tracts, their cytoplasm forming 'cables' (PENFIELD, 1924) along these tracts. Interspersed with these were larger cells resembling oligodendroblasts with paler nuclei which have been described by PENFIELD (1932) as intercalated astrocytes (Fig. 7.) At 20 days the picture had changed substantially. Myelination had started in the corpus callosum. This was more pronounced in the tapetum, the anterior commissure, the fronto-thalamic and thalamo-parietal projections, in cerebellum and superior and middle cerebellar peduncles. The pronounced cellularity previously observed at the lateral margins of the lateral ventricles was substantially reduced, the cerebral mantle showed the beginnings of cellular stratification and laminar organization. The density of cellular elements was noticeably less in between the nerve fibres in the corpus callosum and other tracts, with much fewer immature forms of oligodendroglia, larger numbers of intercalated astrocytic forms, and a decreased tendency to formation of interfascicular glia columns (Figs. 6 and 7). Even at 20 days of age, the mouse brain failed to show a clear-cut distinction between 'grey' and 'white' matter. When this picture was compared with that of the adult mouse brain, the difference in the amount of myelin was most striking (Fig. 6). Study of serial sections confirmed the impression that the 20 day mouse brain possessed not more than one fifth of the total myelin of the adult. That this difference between the 20 day brain and the adult was not one based on tinctorial qualities alone was clearly shown by comparison of the calloso-tapetal complex in cross-section in electron micrographs (Figs. 8(a) and 8(b)). The cerebral and cerebellar cortices were well delineated, and separated from underlying white matter which stained intensely with myelin stains. The laminar arrangement of cells in the cortex was complete, but resulted in a markedly reduced cell-density in the cortex as compared with the 10 day or 14 day brain (Fig. 6), with fewer glial elements present. Satellite oligodendrocytes were scattered throughout the cortex, and large numbers of inter-fascicular oligodendroglia and astrocytes (PENFIELD, 1932) were seen between heavily-myelinated fibres of the white matter. The histological picture was substantially the same in the 42 day mouse brain as in the adult. In the cerebellum, where the number of folia increased with maturation, the most striking change, of significance in this study, was the loss of cells from the molecular layer between the seventh day and the adult stage. In the 5 and 7 day cerebellum the zones corresponding to the adult molecular layer were packed with immature glia cells, while the Purkinje cell layer was from two to three cell-layers deep, interspersed with the feathered cells of Fañanas (PENFIELD, 1932). As development proceeded the Purkinje cells were seen gradually to assume their single-layered arrangement, the compact glial cell mass in the molecular zone gradually disappeared, while the cells in the granular layer continued to proliferate, accounting for a much larger cell population in the 20 day animal than in the 10 day animal. It should be pointed out however, that it is doubtful that the changes in the cerebellum influence the overall chemical compositional

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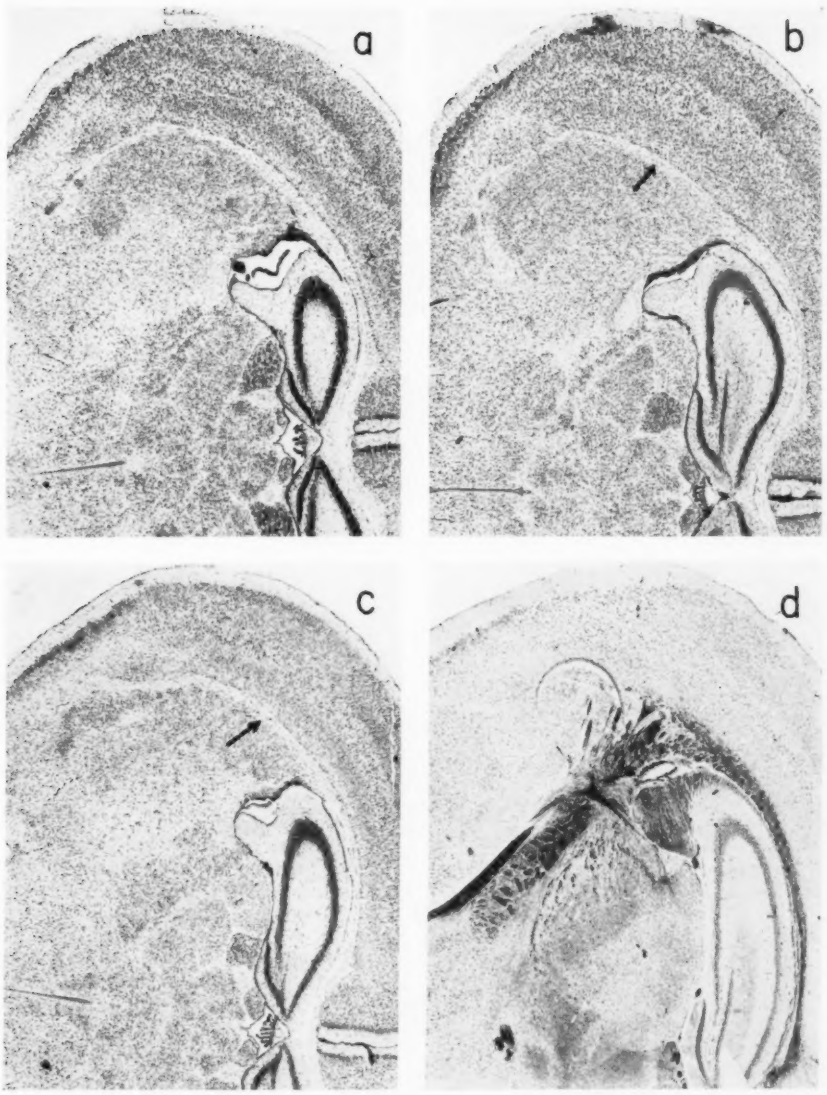

FIG. 6.—Frontal sections through 10 day (a), 14 day (b), 20 day (c) and adult (d) mouse brains at comparable levels. The marked cellularity of the cerebral mantle at 10 and 14 days contrasts with the decrease at 20 days, and still greater diminution in the adult. At 20 days the glial fountains at the lateral walls of the lateral ventricles have greatly diminished, and are absent in the adult. The arrows indicate the heavy columnar arrangement of oligodendroglia and astrocytes in the corpus callosum and tapetum of the 14 day brain in contrast to the relative poverty of these elements, at the same sites, in the 20 day brain. Myelin has just begun to appear in the tapetum in the 20 day brain. Nissl-Luxol Fast Blue staining. ×28.

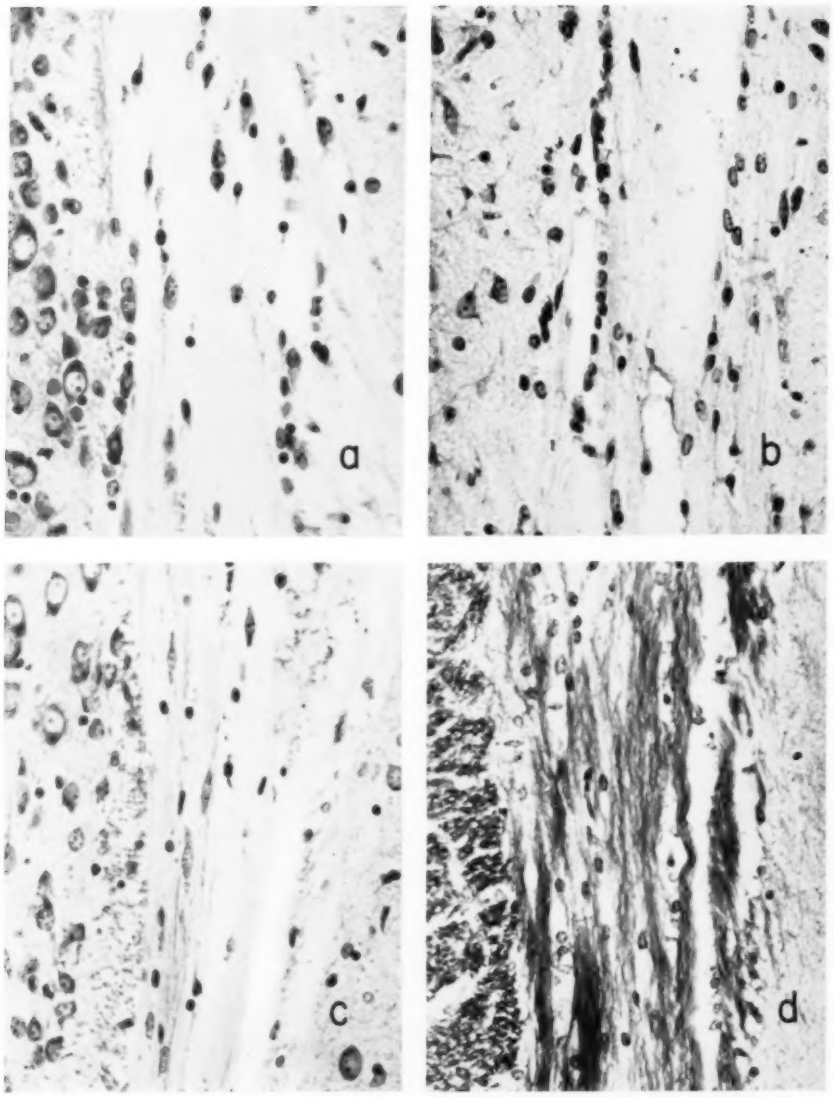

FIG. 7.—Comparison of corpus callosum in frontal section at approximately the same level and the same site in 10 day (a), 14 day (b), 20 day (c), and adult (d) mouse brains. Note the marked increase in the 14 day brain of interfascicular oligodendroglia, oligodendroblasts and astrocytes (arranged in columns) in contrast with the 10 day and 20 day corpus callosum. At 20 days myelination is already evident, while the oligodendroglia are more mature. In the heavily myelinated adult corpus callosum, the interfascicular glia consist of equal numbers of oligoglia and astrocytes. Nissl-Luxol Fast Blue stain. ×400.

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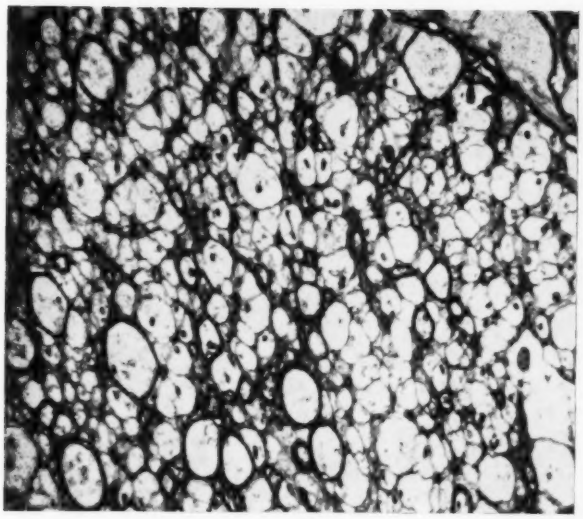
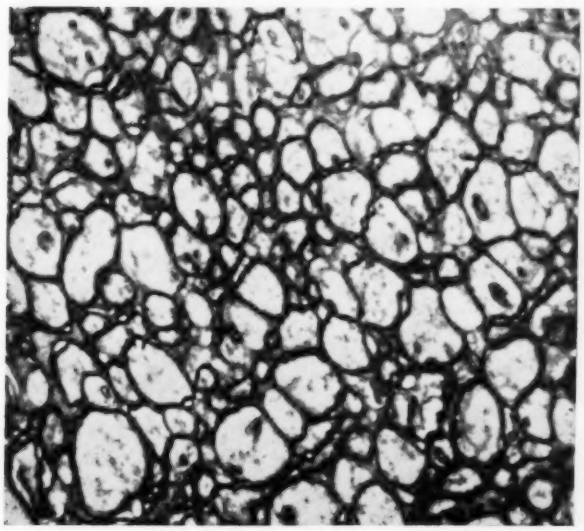


FIG. 8.—(a) Electron micrograph of cross-section of calloso-tapetal fibres in 22 day mouse brain showing the relatively poor degree of myelination as compared with the adult. ×9000.



(b) Electron micrograph of cross-section of calloso-tapetal fibres in adult mouse brain. ×9000.

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changes recorded in this study, taking into account the relative amounts of tissue involved. This would be especially true of cerebellar white matter.

The ground substance, in the course of development of the mouse brain, showed an increasing affinity for PAS-staining with increasing age of the brain, being maximal in the adult. The neuraminic acid stain with Bial's reagent, as used by DIEZEL (1955) gave a picture identical to that obtained with PAS. In both instances the visible glial processes were not stained, nerve cell bodies stained faintly while the ground substance showed maximal staining. In this respect, our findings confirm fully the earlier observations of HESS (1953, 1955).

DISCUSSION

The rapid changes taking place in the composition of the immature mouse brain render it imperative to relate analytical results to several parameters determined for each age if the results are to be amenable to interpretation. This necessitates the expression of results both in terms of absolute amounts as well as in percentages of chosen constituents. We have endeavoured to present our data in a form where both kinds of information are available to the reader. The difficulty of correct expression becomes evident when one considers the data presented here on the decrease in brain weight occurring between 16 and 23 days. This decrease is clearly the result of loss of water, inasmuch as the absolute amount of total solids (dry weight) per brain continues to rise (Table 1). It appears remarkable that this point has not previously been adequately emphasized because the very rapid loss of water preceding the very active period of myelin deposition was fully described as early as 1913 by KOCH and KOCH (1913) and subsequently by DONALDSON (1916) for the 20 day period of the albino rat. Thus the decrease of brain weight observed by us between the 16 and 23–24 days in the mouse appears almost unavoidable if one considers that a 10 per cent loss of water would have to be compensated for by approximately a 30 per cent increase in dry weight in order that no loss in total brain weight be recorded within the same period. The fallacious impression that would be created by expressing brain constituents as a percentage of fresh weight is thus quite evident. The loss of water from the immature mouse brain was preceded by a sharp fall in absolute content of desoxyribose nucleic acid. Since this loss was also reflected in a drop in the lipid-free total residue (Fig. 2) one may conclude that the loss of water at this stage of the development is accompanied by a decrease in cell population. Confirmation of this is found in the relative poverty of glial elements in the 20 day brain as compared with the 14 day mouse brain. The reason that there is no similar decrease of RNA at that time is probably due to the summation of two factors: the cells that are lost do not possess any significant amount of RNA in their cell processes, and the fact that the processes of remaining nerve and glia cells are constantly growing and adding RNA thus increasing the amount of RNA content of each developing brain.

Most of our data on the lipid constituents of developing mouse brain are in essential agreement with those reported by FOLCH (1955). However, the additional information provided by data on DNA and RNA content and changes in water-soluble glycolipid composition when correlated with the lipid analyses for the same age groups permit the interpretation of the chemical findings with respect to the morphological changes observed in our detailed histological study.

At a time when histologically recognizable myelin constitutes a negligibly small

portion of the brain (1–14 day period) our data shows a sixfold increase in total lipid (with a proportional increase in the phosphatide fraction) with a parallel increase in total residue, fresh weight and nucleic acid (Fig. 2 and Table 1). Thus, although the 14 day brain possesses over 60 per cent of its adult complement of total lipid and almost half its complement of cerebrosides (Fig. 5), a proportional amount of myelin is not in evidence. It seems reasonable to conclude that the major portion of this lipid should be ascribed to cell-body and cell-process lipids. The 14–23 day period in which there is no significant increase in cerebrosides (Fig. 2, 5 and Table 1) marks the stage during which histologically recognizable myelin becomes very much in evidence in all the major tracts (Fig. 6, Fig. 8(a)). The most striking additions to the whole myelin content of the brain occur in the 23 day to adult stage (Figs. 6, 7, 8(a) and 8(b)). Yet, the adult brain was found to have only a 22 per cent higher total lipid content than the 20 day brain and only a 40 per cent higher content in cerebrosides. We find it difficult to reconcile these findings with previous concepts based on the assumption that myelination involves the elaboration or acquisition of additional lipid in proportion to the amount of myelin layed down by the developing brain.

An alternate explanation would be that myelination involves to a great extent the re-orientation of existing lipids, already present in cells, cell-processes and cell-membranes of brain, into distinct structural units which thereby acquire the properties by which myelin is defined and recognized (FERNANDEZ–MORAN, 1954; GEREN and SCHMITT, 1955). We interpret our findings to be consistent with this explanation. The absolute increase in the cerebroside content of the brain observed by others (KOCH and KOCH, 1913; FOLCH-PI, 1955; BRANTE, 1949) and by us, may indicate that cerebrosides play a key role in rendering this process possible.

The compositional changes in water-soluble glycolipids occurring at all ages appear to reflect changing proportions of neuraminic acid containing fractions (gangliosides and strandin) and fractions poor in neuraminic acid content (polycerebrosides). Up to the tenth day the latter appear to predominate, while from the twelfth day on a greater portion of the water-soluble glycolipid fraction is represented by gangliosides. The points which appear of significance are that (a) up to the seventh day the water-soluble glycolipids represent the sole glycolipids of mouse brain, cerebrosides being absent, as was also shown by FOLCH-PI (1955); (b) after the sharp increase of neuraminic acid about the twelfth day the water-soluble glycolipid fraction continues to show compositional fluctuations with advancing maturation; and (c) the water-soluble glycolipids occur in about the same amount in the 12 day brain as in the adult (Fig. 5). The last point makes it highly likely that after the twelfth day the water-soluble glycolipids constitute a reservoir, constantly replenished and constantly drawn upon for other purposes. This, in turn, would imply a high turnover rate as has already been shown to exist in the adult animal by RADIN *et al.* (1957). The data presented on the changes in neuraminic acid content, reducing power before hydrolysis, and total hexose content in relation to the progressive increase in cerebroside content of the brain suggest the likelihood that the water-soluble glycolipids may constitute the precursors from which cerebrosides are derived.

SUMMARY

The developing mouse brain has been studied from 1 day to the adult stage (10 weeks) with respect to changes taking place in total lipid, total lipid-free residue,

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total water, total lipid phosphorus, total nucleic acids (RNA and DNA), cerebrosides and water-soluble glycolipids (gangliosides and polycerebrosides), in an effort to delineate the role played by water-soluble glycolipids in the early phases of myelination. An attempt was made to correlate these changes with the changing histological picture and the development of myelin. The data obtained have led to the following conclusions:

(1) The onset of histologically evident myelination is preceded by a sharp drop in water and nucleic acid content of the brain, presumably due in part, to a decrease in glial cellular elements.

(2) Although the increase in cerebrosides parallels the increase in myelination, this increase is not accompanied by a proportional net increase in other lipids, so that the bulk of myelin-lipids appears to be formed from lipids already present in brain when myelin makes its appearance and continues to increase.

(3) Water-soluble glycolipids show a steady increase until the twelfth day, and from then on maintain a relatively constant absolute level in spite of the continued growth and maturation of brain. This suggests a high rate of turnover for this group of substances.

(4) In the 1–12 day period the water-soluble glycolipid fraction is poor in neuraminic acid (polycerebrosides predominate) while from then on a relatively constant proportion of gangliosides and polycerebrosides is in evidence.

The results are discussed in relation to the manner in which myelin may be formed, and with respect to the possible role of water-soluble glycolipids as precursors of cerebrosides.

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THE EFFECTS OF 2-DEOXY-D-GLUCOSE ON METABOLISM OF SLICES OF CEREBRAL CORTEX INCUBATED *IN VITRO*

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PREVIOUS investigations have characterized 2-deoxy-D-glucose* as an inhibitor of glucose metabolism. Its effectiveness in inhibiting glycolysis and growth of neoplastic tissues (ELY, 1954; WOODWARD and HUDSON, 1954; SOKOLOFF *et al.*, 1956; BALL, WICK and SANDERS, 1957) suggested clinical applications. During evaluations of its toxicity in animals preliminary to such trials, LANDAU and LUBS (1958) observed neurological signs and symptoms which closely resembled those of insulin-induced hypoglycaemia.

After oral or parenteral administration, 2-deoxyglucose is rapidly distributed throughout the body and appears promptly in the cerebrospinal fluid (WICK, DRURY and MORITA, 1955; LANDAU and LUBS, 1958). Using doses of the order of 12 m-moles/kg, LANDAU and LUBS (1958) found an elevation of blood glucose to levels two or three times normal control values. Under such conditions both blood glucose and 2-deoxyglucose respond to insulin (WICK *et al.*, 1955), but the insulin-mediated decrease in blood glucose to normal ranges intensifies the apparent hypoglycaemic picture. These observations are consistent with inhibition of tissue utilization of glucose in the presence of 2-deoxyglucose, resulting in a situation of simultaneous *hyper*-glycaemia and cytoglycopenia, which in the central nervous system is expressed as dysfunctions usually associated with hypoglycaemia.

2-Deoxyglucose is readily phosphorylated by yeast, muscle and brain hexokinase (SOLS and CRANE, 1954; WOODWARD and HUDSON, 1955; WICK *et al.*, 1957), and it produces inhibition of glycolysis in yeast and various mammalian tissues, including brain (WOODWARD, 1952; WOODWARD and HUDSON, 1954). Metabolism of 2-deoxy-glucose-6-phosphate appears not to occur (SOLS and CRANE, 1954; WICK *et al.*, 1957) and in purified kidney preparations it has been reported to block conversion of glucose-6-phosphate to fructose-6-phosphate, with possible secondary effects on hexokinase (WICK *et al.*, 1957).

Few studies of the effects of 2-deoxyglucose on cerebral metabolism have been reported. Anaerobic glycolysis of rat cerebral cortex slices is inhibited 50 per cent when incubated with 1 mM-2-deoxyglucose plus 15 mM-glucose *in vitro*, a result comparable to those obtained with rat diaphragm or with tumour tissues under similar conditions, but oxygen uptake by rat cerebral cortex appears much less

* Hereafter referred to as 2-deoxyglucose or 2-DG.

sensitive, concentrations of 2-deoxyglucose up to 60 mM being required to achieve 50 per cent inhibition (WOODWARD and HUDSON, 1954). In studies on brain hexokinase, SOLS and CRANE (1954) found that 2-deoxyglucose is phosphorylated at a rate comparable to that of glucose but that the phosphate ester formed is not a substrate for either glucose-6-phosphate dehydrogenase or phosphohexoseisomerase.

Our interest in the effects of 2-deoxyglucose stem from use of incubated slices of cerebral cortex for investigation of various facets of cortical metabolism. As a whole cell preparation with relatively intact and integrated metabolism, slices offer certain advantages in a number of instances, but the metabolic systems concerned are usually complex and difficult to dissect into their component parts. The use of metabolic inhibitors can be of assistance here, provided that their mechanism of action can be clearly established. Preliminary data on 2-deoxyglucose, reviewed above, seemed to be promising in this regard. Accordingly, the following investigations were undertaken with three aspects in mind: (a) to explore more fully the mechanisms of action of 2-deoxyglucose on cerebral cortex metabolism; (b) to utilize 2-deoxyglucose to delineate the characteristics of glucose metabolism in cerebral cortex, particularly in respect to the relative importances of the Embden-Meyerhof glycolytic pathway and the Warburg–Dickens–Lipmann oxidative pathway; and (c) to investigate effects of 2-deoxyglucose on several systems in cerebral cortex presumed to be dependent upon glucose metabolism for their maintenance and function.

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EXPERIMENTAL

Materials. Stock, adult cats were used throughout. Reagent grade 2-deoxy-D-glucose and 3:5-diaminobenzoic acid were obtained from the Aldrich Chemical Co., Milwaukee, Wis. Adenosine triphosphate (sodium) and glucose-6-phosphate (sodium) were obtained from the Sigma Chemical Co., St. Louis, Mo., and fructose-6-phosphate (barium), ribose-5-phosphate (barium) and partially purified yeast hexokinase from Nutritional Biochemicals Corp., Cleveland, Ohio. [^{14}C]-D-Glucose was obtained from Nuclear-Chicago Corp., Chicago, Ill., and [1-^{14}C]-D-glucose and [6-^{14}C]-D-glucose were kindly donated by Dr. R. O. BRADY of our Institute. Hyamine base and toluene liquid scintillation counting fluid were generously supplied by Dr. B. W. AGRANOFF of our Institute. All other compounds were of reagent grade, obtained from usual commercial sources.

Preparation of 2-deoxy-D-glucose-6-phosphate. 20 μmoles of 2-deoxy-D-glucose in 0·5 ml 25 mM-$NaHCO_3$ were mixed with 2·0 ml of 25 mM-$NaHCO_3$ containing 50 μmoles of $MgCl_2.6H_2O$ and 60 K.M. units of yeast hexokinase in Warburg vessels. Sodium ATP, 50 μmoles in 0·5 ml 25 mM-$NaHCO_3$, was placed in side bulbs, and the vessels gassed with 95% N_2–5% CO_2. After equilibration, the ATP was tipped in and incubation at 37° carried out for 30 min. Vessel contents were added to 2·0 ml of 5% trichloroacetic acid, centrifuged, and the supernatant shaken with acid-washed Darco G charcoal to remove excess ATP and filtered. The filtrate was treated with 0·5 ml 25 per cent barium acetate, centrifuged to remove inorganic phosphate, and the supernatant brought to pH 7·5 with 5 N-KOH and treated with 4 vol. of 95% ethanol. After standing 12 hr at 0–4°, the barium salt precipitate was recovered by centrifugation, air dried, and dissolved in 1·0 ml 0·1 N-HCl. The solution was shaken with Amberlite IR-120 cation exchange resin (H^+ form) to remove Ba^{++} and the solution separated from the resin by capillary suction pipettes, neutralized with N-KOH and

preserved in the frozen state. Samples of glucose-6-phosphate and fructose-6-phosphate were prepared simultaneously in identical fashion. Analyses for unreacted substrates indicated conversion was better than 75 per cent. The author is indebted to Dr. G. G. ASHWELL, Laboratory of Biochemistry and Metabolism, National Institute of Arthritis and Metabolic Diseases, for valuable suggestions in design of the above procedure.

Preparation of bacterial enzyme for glutamate assays. A lyophilized culture of *Clostridium perfringens* (Welchii), type A, strain SR-12 (American Type Culture Collection No. 8009), was dissolved in 0·4 ml of broth from a beef liver medium for anaerobes, transferred quantitatively to the meat tube and cultured for 48 hr at 37°. This was subcultured by transfer of 3 ml of broth to flasks containing 100 ml of medium recommended by MEISTER, SOBER and TICE (1951) with incubation for 30 hr at 37°. Flask contents were added to 10 l. of the same medium and incubated an additional 48 hr. Bacteria were harvested with a Sharpless continuous centrifuge, washed with 0·9% NaCl, and lyophilized. The yield per 10 l. was approximately 10 g of bacteria (wet weight). Activity of the final preparation was assayed by the procedure of KREBS (1948) as modified by MEISTER *et al.*, (1951), using L-glutamine and L-glutamic acid as substrates to check both deamidase and decarboxylase potencies.

METHODS

Incubation procedures. Unanaesthetized cats were sacrificed by decapitation and brains removed within 5–7 minutes. They were placed in a humid chamber (ELLIOTT, 1946) and slices of cerebral cortex cut without moistening at 0·45 mm using a fresh tissue microtome (STADIE and RIGGS, 1944). Mixed pairs of slices, weighing usually 150–200 mg, were suspended in appropriate incubation media (2·0 or 3·0 ml final volume) in Warburg type vessels. Incubation media of the following composition in m-moles/l. final concentration were used:

(a) A 3 mM-K^+ bicarbonate medium (TOWER and ELLIOTT, 1952): NaCl, 122; KCl, 3; $CaCl_2$, 1·3; $MgSO_4$, 1·2; KH_2PO_4, 0·4; $NaHCO_3$, 25 and glucose, 10.

(b) A 27 mM-K^+ bicarbonate medium (TOWER and ELLIOTT, 1952): Same as (a) except for NaCl, 98 and KCl, 27.

(c) A 27 mM-K^+ phosphate medium (ELLIOTT and HENDERSON, 1948): NaCl, 98; KCl, 27; $MgSO_4$, 1·2; KH_2PO_4, 0·4; Na_2HPO_4 (pH 7·4), 17·5 and glucose, 10. Vessels containing bicarbonate media (a or b) were gassed with 95% O_2–5% CO_2 for aerobic and 95% N_2–5% CO_2 for anaerobic experiments. The latter gas mixture was rendered oxygen-free by passing it over hot copper filings, as outlined by ELLIOTT (1949). For phosphate medium (c) 100% O_2 constituted the gas phase, and double side bulb vessels were used, containing 0·2 ml of M-Hyamine base as CO_2 absorbant in the centre well (PASSMAN, RADIN and COOPER, 1956) and 0·3 ml 2N-H_2SO_4 in the second side bulb. Dixon–Keilin vessels contained 0·3 ml 3N-HCl in the side bulbs and 0·2 ml 15% KOH in centre well cups with filter paper wicks and glass displacement rods in the centre wells.

Substrates under study were placed in side bulbs dissolved in aliquots of medium, including additional potassium to give 27 mM (TOWER and ELLIOTT, 1952), except in the case of Dixon–Keilin vessels where all media and substrates were placed in the main vessel compartment. All incubations were carried out at 37° on a Warburg-type bath. After 5–10 min equilibration on the bath, side bulb contents (medium and substrate) were added to the main compartment. Initial control samples were removed immediately and incubation samples continued for 1 hr unless otherwise specified. In most cases incubation was terminated by immediate icing of vessels. Slices and media were separated by suction filtration through No. 54 Whatman filter paper discs (TOWER and ELLIOTT, 1952) and were appropriately preserved for assays. Where indicated, incubation was terminated by addition of acid from side bulbs into the incubation mixture.

Differential manometry for determination of aerobic glycolysis, oxygen uptake and respiratory quotient on the same sample was carried out using Summerson differential manometers and Dixon–Keilin vessels, according to the procedure developed by DIXON and KEILIN (1933) and SUMMERSON (1939).

Analytical. Unless specified otherwise all colorimetric readings were made with a Coleman Model 14 Spectrophotometer.

(a) *Glucose.* The method of NELSON (1944) was employed. Filtrates (1 : 10) from 1·0 ml aliquots of incubation medium were prepared, using 5% $ZnSO_4$ and 0·3 N-NaOH (instead of $Ba(OH)_2$). Otherwise NELSON's procedure was followed exactly. Tissue slices, homogenized in distilled water, were treated in similar fashion for 'free' tissue glucose. Under these conditions the presence of 2-deoxyglucose interferes, equimolar concentrations of 2-deoxyglucose giving readings at 520 mμ approximately 20 per cent of those obtained with glucose. Correction to true glucose values was made by running standards with and without added 2-deoxyglucose on each occasion and determining 2-deoxyglucose contents directly on aliquots of each sample.

(b) *2-Deoxyglucose.* The procedure of CRAMER and NEVILLE (1953) was applied to aliquots of the same $ZnSO_4$–NaOH filtrate used for glucose assays. The presence of glucose does not affect this procedure and authentic 2-deoxyglucose-6-phosphate gives no interference. The quinaldine reagent for the test was prepared as follows: 1·1 g 3:5-diaminobenzoic acid was dissolved in 50 ml hot distilled water, decolourized with Norit A charcoal, and filtered. Concentrated HCl (6 ml) was added to bring the pH to about 2, then 25 ml 85% phosphoric acid was added, and the solution made to 85 ml with distilled water. A second decolorization with Norit A was carried out and the resulting clear, colourless reagent stored in a dark bottle. Satisfactory standard curves for concentrations of 2-deoxyglucose between 0·25 and 2·5 μM initial aliquot content were obtained, reading at 420 mμ.

(c) *Lactic acid.* Aliquots of the $ZnSO_4$–NaOH filtrate (for glucose) were used for lactic acid assays by the method of BARKER and SUMMERSON (1941). It was found that the Cu–$Ca(OH)_2$ precipitation procedure described by them would completely remove ^{14}C-labelled glucose from incubation media $ZnSO_4$–NaOH filtrates, so that ^{14}C-activity in lactic acid derived from metabolized [^{14}C]-glucose could be counted alone in the supernatant after such precipitation. In this way accurate counts of total ^{14}C-activity (glucose + lactate) and of [^{14}C]-lactate only in the same sample of medium could be readily obtained.

(d) *Acid-soluble phosphates.* Incubated tissue slices were homogenized in 5·0 ml ice-cold 10% trichloroacetic acid, centrifuged at 0° and the supernatant fluid analysed for acid-soluble phosphates by one or both of the following procedures:

(i) *Without barium precipitation.* The method of ERNSTER, ZETTERSTRÖM and LINDBERG (1950) was employed. Two aliquots of each supernatant sample were used. One was hydrolysed with 0·5 ml 10 N-H_2SO_4 for 20 min on a boiling water bath for total labile and inorganic phosphate. The other while ice-cold was extracted with *iso*butanol-benzene for inorganic phosphate, and then, after standing at room temperature for 30 min, re-extracted for phosphocreatine phosphate (HEALD, 1954). Other labile acid-soluble phosphates were obtained by difference.

(ii) *Barium-alcohol fractionation.* Alternatively the acid-soluble supernatant was subjected to the fractionation procedure of LEPAGE (1949) with applications to brain tissue suggested by HEALD (1954, 1956) to obtain a barium-insoluble and a barium-soluble, alcohol-insoluble fraction. Each of the precipitated fractions was dissolved in a minimum volume of 0·1 N-HCl, the Ba^{++} removed by shaking with Amberlite IR-120 cation exchange resin (H^+form), the supernatant suctioned off with capillary pipettes and made to 4·5 ml with distilled water. Two aliquors of each fraction for each sample were then run by procedure (i) for inorganic phosphate, creatine phosphate and total labile and inorganic phosphates. With normal slices of cerebral cortex the two procedures run in parallel on the same sample gave identical results for creatine phosphate and inorganic phosphate. Adenosine polyphosphates isolated by procedure (ii) corresponded closely to the residual labile phosphate in procedure (i) after subtraction of creatine phosphate from the total.

(e) *Glycogen.* The acid-insoluble residue from trichloroacetic acid extraction of slices was used for glycogen determinations by the method of LEBARON (1955). Glucose liberated was assayed by the method of NELSON (1944), using a 6·0 ml final volume for reading, and parallel assays for 2-deoxyglucose were run on the hydrolysate by the method of CRAMER and NEVILLE (1953).

(f) *Glucose-6-phosphate.* Tissue slices were treated as for acid-soluble phosphates. The trichloroacetic acid supernatant was extracted with ethyl ether to remove the trichloroacetic acid, brought to pH 6–7 with 5 N-KOH and assayed enzymatically by the methods of KORNBERG and HORECKER (1955) and HORECKER and WOOD (1957). The author is indebted to Dr. G. G. ASHWELL for carrying out these assays.

(g) *Non-nucleotide pentoses.* Aliquots of the trichloroacetic acid supernatant, as above, were

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shaken with acid-washed Darco G charcoal (suspension in 1 N-HCl, filtered and dried) to remove nucleotides, using 20 mg of charcoal per estimated μmole of nucleotides present, and filtered. The efficacy of removal was controlled by examining samples at 260 mμ in a Beckman DU Spectrophotometer before and after charcoal treatment. Total non-nucleotide pentoses were determined by the method of MEIJBAUM (1939), reading at 660 mμ.

(h) *Paper chromatography of phosphorylated carbohydrates.* Trichloroacetic acid extracts, as prepared for acid-soluble phosphates, were neutralized with 1 N-KOH, treated with 1·0 ml 25% barium acetate and 4 vols. of 95% ethanol, and allowed to stand overnight at 0–4°. The dried barium precipitates were dissolved in 0·2 ml 0·1 N-HCl, shaken with Amberlite IR-120 cation exchange resin (H^+ form) to remove Ba^{++}, and the supernatant fluid neutralized with 1 N-KOH. Duplicate one-dimensional paper chromatograms were prepared, using 50 μl aliquots (=50 mg original tissue) of the above final solution. Chromatograms were developed descending with *n*-amyl acetate-glacial acetic acid-water (3 : 3 : 1 v/v/v) as solvent for 36–48 hr (ASHWELL, 1957). Positions of hexose and pentose phosphates were visualized in each of two ways—phosphate moieties by the ferric chloride-sulphosalicylic acid reagent of WADE and MORGAN (1953) and carbohydrate moieties by the *p*-anisidine-HCl reagent of HOUGH, JONES and WADMAN (1950).

(i) *Glutamic acid and glutamine.* Incubation media and tissue slices were assayed microbiologically by the method of KREBS (1948) as modified by MEISTER *et al.* (1951), using the *Clostridium perfringens* preparation, for total glutamate (glutamic acid + glutamine). Tissues and media were prepared as recommended by STERN *et al.* (1949). Assays were carried out manometrically in the presence of 6 mM-cetyltrimethylammonium bromide (Cetavlon), as recommended by MEISTER *et al.* (1951). Aliquots of each sample were run simultaneously without added bacterial reagent to serve as tissue controls, together with bacterial and thermobarometric control vessels. Amide ammonia liberated from glutamine was assayed, together with tissue, bacterial and reagent controls, by the CONWAY (1947) microdiffusion technique as applied by RUSSELL (1944) for colorimetric analysis. For the latter the contents of the centre well of the Conway units were quantitatively transferred with capillary pipettes to Benedict–Nash tubes calibrated at 6·0 ml, the centre wells being rinsed successively with 1·0 ml 0·001 N-HCl and 1·5 ml ammonia-free water. Glutamic acid was taken as the difference between total glutamate (manometric) and glutamine (amide ammonia).

(j) *Electrolytes.* Tissue slices were homogenized in deionized water, centrifuged and the supernatant fluids made up to appropriate volume for analysis. Aliquots (1·0 ml) of incubation media suitably diluted with deionized water were used. Sodium and potassium were determined with a Perkin–Elmer model 52C flame photometer, using the lithium internal standard method. Chlorides were determined on 0·1–0·2 ml aliquots of incubation media or slice extracts by the amperometric method of COTLOVE *et al.* (1958).

(k) *Carbon-14 studies.* With the exception of $^{14}CO_2$, all ^{14}C counts were made with a Nuclear-Chicago D-47 gas flow counter assembly. All samples were counted for 10,240 counts with conditions standardized by counting all samples (tissue or media) on Whatman No. 1 filter paper circles in counting planchets. All samples were at infinite thickness and were placed to ensure comparable geometry. Overall counting efficiency by this technique was calculated to be about 10 per cent.

Tissue fractionation for ^{14}C-labelled hexose and pentose phosphates was carried out chromatographically, as outlined above, counts being made on each spot visualized. For ^{14}C-labelled amino acids and other constituents, tissue slices were homogenized in 4·0 ml 75% ethanol, centrifuged and the supernatants evaporated with gentle heat to 0·5 ml. Incubation media were used direct. 50 μl aliquots (=30 mg original tissue) were chromatographed in two dimensions on Whatman No. 1 paper. The chromatograms were developed descending with water-saturated phenol in the first dimension, followed by 2 : 6-lutidine–ethanol–water (55 : 20 : 25 v/v/v) in the second. This procedure is similar to that of ROBERTS and FRANKEL (1949). Radioautograms were prepared of each developed chromatogram, using Eastman no-screen X-ray film exposed in regular X-ray cassettes for from 1 to 4 weeks. Amino acids were then visualized by ninhydrin spraying, and counts made on each spot located radioautographically or visually.

$^{14}CO_2$ was determined after absorption into Hyamine base contained in centre wells of incubated vessels, as outlined previously. The Hyamine base was transferred quantitatively to counting vials and the centre wells rinsed with a total of 1·0 ml of liquid scintillation counting fluid (AGRANOFF 1957). Each sample vial was made up to a final composition of 5·0 ml of counting fluid and 0·4 ml

Hyamine base (the additional 0·2 ml clarifying any absorbed water in the sample). The radioactivity of each sample was counted with a Packard Liquid Scintillation counter. Variations in count due to non-homogeneity of samples were corrected by addition of a standard aliquot of [^{14}C]-palmitic acid (toluene soluble) of known activity to each sample, recounting and calculating the μc content of each unknown. The author is indebted to Drs. B. W. AGRANOFF and R. O. BRADY of our Institute for advice in setting up this method, for generous gifts of reagents, and for the use of their counter.

RESULTS AND DISCUSSION

Glycolysis and oxygen uptake

Slices of cat cerebral cortex were incubated in a bicarbonate-saline medium containing 10 mM-glucose with either 3 mM or 27 mM-potassium. Aerobic glycolysis, oxygen uptake and respiratory quotient were determined on the same sample by differential manometric techniques, and anaerobic glycolysis was determined by simple manometry. Results are summarized in Table 1. The average values compare well

TABLE 1—MANOMETRIC CONTROL VALUES FOR INCUBATED SLICES
(μmoles/g per hr)

K^+ conc	No.	O_2 uptake	RQ	Aerobic glycolysis	Anaerobic glycolysis
27 mM	6	−85·0 (±4·0)	0·92 (±0·03)	52·1 (±3·0)	—
	2	—	—	—	24·8 (±0)
3 mM	2	−71·8 (±3·3)	0·96 (±0·02)	35·2 (±1·8)	—
	6	—	—	—	50·4 (±4·0)

Standard deviations given in parentheses.
Aerobic studies by the differential manometric technique (DIXON and KEILIN, 1933; SUMMERSON, 1939). See text for details.

with those reported for cat cortex studied under similar conditions by ELLIOTT and HENDERSON (1948). The well known effects of potassium ion concentrations on aerobic and anaerobic glycolysis are clearly illustrated.

The results of adding various concentrations of 2-deoxyglucose to the incubation medium of such slices are shown in Fig. 1. For these and subsequent studies all aerobic experiments were carried out with 27 mM-potassium, and all anaerobic experiments with 3 mM-potassium, to take advantage of the respective higher rates of glycolysis under these conditions (Table 1). However, identical effects of 2-deoxyglucose were obtained regardless of which potassium concentration was used. Under aerobic conditions glycolysis is inhibited 50 per cent by 4 mM-2-deoxyglucose and 75 per cent by 10 mM-2-deoxyglucose. At the latter concentration oxygen uptake is inhibited only about 30 per cent, but the respiratory quotient (RQ) in these experiments averaged about 0·8, compared with 0·92 for controls. Under anaerobic conditions, glycolysis is inhibited 50 per cent by approximately 0·8 mM-2-deoxyglucose. This value is very close to the 1·0 mM-concentration reported by WOODWARD and HUDSON (1954) for 50 per cent inhibition of anaerobic glycolysis of rat cerebral cortex slices. These investigators did not study aerobic glycolysis but did report inhibition of rat cortex oxygen uptake to reach 50 per cent with about 60 mM-2-deoxyglucose.

Calculations of the slopes of the inhibition curves for anaerobic and aerobic

glycolysis shown in Fig. 1 indicate that they are virtually parallel. The explanation of the fivefold greater sensitivity of cerebral cortex to a given concentration of 2-deoxyglucose under anaerobic conditions is not entirely apparent. On the basis of their findings, WOODWARD and HUDSON (1954) suggested that higher levels of adenosine triphosphate (ATP) may be maintained under aerobic conditions and thus provide a greater degree of protection against 2-deoxyglucose. This suggestion seems to be a reasonable explanation for part of the difference observed, but, if it is the complete explanation, it would indicate a very narrow range of available ATP under anaerobic conditions.

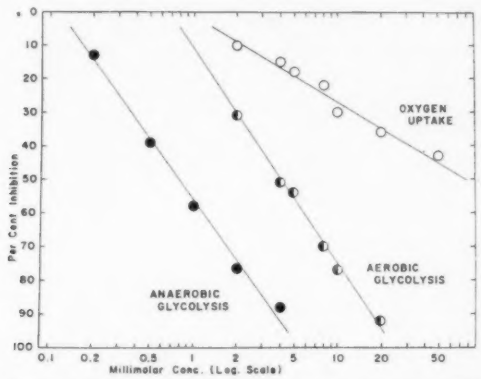

FIG. 1. Effect of concentration of 2-deoxyglucose upon oxygen uptake, aerobic glycolysis and anaerobic glycolysis of incubated slices of cat cerebral cortex. Each point plotted is the mean of three or more determinations with standard deviations of not more than ±5 per cent of any mean value. The theoretical curves were calculated from individual values by the method of least squares with standard deviations of ±5 per cent for each regression coefficient. Aerobic studies were carried out with media containing 27 mM-K^+; anaerobic studies with 3 mM-K^+ (see text for details).

On the basis of results in Fig. 1, subsequent investigations have been carried out with 10 mM-2-deoxyglucose aerobically and with 1 mM-2-deoxyglucose anaerobically. These concentrations produce average inhibitions of 75 per cent and 55 per cent respectively.

Glucose and 2-deoxyglucose metabolism

The manometrically determined values for aerobic glycolysis in control slices and those incubated with 10 mM-2-deoxyglucose were confirmed by chemical determination of lactic acid production. Average values are given in Table 2. Lactate production by

TABLE 2—EFFECT OF 10 mM-2-DEOXYGLUCOSE ON GLUCOSE UTILIZATION BY INCUBATED SLICES (AEROBIC; 27 mM-K^+) (μmoles/g per hr)

	Glucose uptake	Lactic acid production	2-Deoxyglucose uptake
Control (8)	−43·6 (±2·9)	45·5 (±4·6)	
10 mM-2-Deoxyglucose (8)	−24·7 (±2·3)	11·9 (±1·3)	−14·7 (±2·9)

Numbers of determinations and standard deviations given in parentheses.

control slices correspond closely to manometric values given in Table 1, the difference being accounted for largely by the small percentage in tissue slices determined manometrically but not by lactate analyses on incubation media only. The degree of inhibition of lactate production by 10 mM-2-deoxyglucose is of the same order by both methods. Lactate analyses for slices incubated at other concentrations of 2-deoxyglucose showed a similar correspondence.

Under these conditions the *gross* uptake of glucose by incubated cortical slices is markedly inhibited by the presence of an equimolar concentration (10 mM) of 2-deoxyglucose. A considerable *gross* uptake of 2-deoxyglucose occurred in these same slices. It should be emphasized that these are gross values since they have not been corrected for any unmetabolized 'free' glucose or 2-deoxyglucose in tissue slices. The importance of this qualification is illustrated by the data in Table 3. Control slices

TABLE 3—GLUCOSE AND DERIVATIVES IN INCUBATED SLICES
(μmoles/g)

	Initial	60 min	60 min + 2-DG (10 mM)
Free glucose (3)	5·1 (±0·8)	2·95 (±1·25)	9·2 (±0·4)
Free 2-deoxyglucose (3)	—	—	10·75 (±4·9)
Glycogen (4)	0·75 (±0·4)	0·85 (±0·4)	0·85 (±0·2)
Glucose-6-phosphate (2)	<0·35	<0·6	<0·6
Non-nucleotide pentoses (total) (2)	1·55	1·4	1·5

Numbers of determinations and standard deviations given in parentheses.

contained only about 7 per cent of the total uptake as 'free' unmetabolized glucose. A comparable value was reported by BELOFF-CHAIN *et al.*, (1955) for rat cerebral cortex slices. In contrast, slices incubated with 10 mM-2-deoxyglucose contained some 40 per cent of the total glucose taken up still as 'free', unmetabolized glucose. A comparable concentration of unmetabolized 2-deoxyglucose was present in these slices. These findings are consistent with previous *in vivo* observations that in the presence of 2-deoxyglucose, glucose reaches tissues but is not utilized by them (NAKADA and WICK, 1956; LANDAU and LUBS, 1958).

Since WICK *et al.* (1957) had suggested that the primary mechanism of action of 2-deoxyglucose appeared to be a block of the glucose-6-phosphate to fructose-6-phosphate step, evidence in support of this point in incubated cortical slices was sought, as shown in Table 3. No differences in glycogen levels of control and 2-deoxyglucose-inhibited slices were found. These levels are of the same order as those previously reported by LEBARON (1955). On hydrolysis of glycogen from slices incubated with 2-deoxyglucose no detectable 2-deoxyglucose was present in this fraction. No increase in slice levels of glucose-6-phosphate were evident when cortical slices were incubated with 2-deoxyglucose, nor were the levels of non-nucleotide total

pentoses in such slices increased. The findings in Table 3 provide no evidence for an accumulation of intermediates in the pathways available as alternates to glycolysis, but they do not necessarily disprove the presence of a postulated block in the glycolytic sequence.

TABLE 4—DISTRIBUTION OF ^{14}C-LABELLING IN SLICES OF CAT CEREBRAL CORTEX INCUBATED WITH [^{14}C]-D-GLUCOSE

	Controls	10 mM-2-DG
% Total counts/min utilized:	48·4	33·3
% Total counts/min 'free' in tissues:	3·7	18·7
Net % metabolized:	44·7	14·6
[Calculated μmoles/g per hr. metabolized*]	[40.9]	[13·4]
% Total metabolized in		
Lactic acid	70·0	59·0
CO_2	21·5	31·0
Free amino acids	7·2	7·0
Other components	1·3	3·0
% Total free amino acid fraction as		
Glutamic acid	45·0	49·0
Glutamine	20·0	14·5
γ-Aminobutyric acid	10·0	8·5
Aspartic acid	18·0	14·0
α-Alanine	6·5	13·0
Serine	0·5	0
% Total in other components		
Glycogen	6·2	8·0
Glucose-6-P	9·4	5·2
Fructose-6-P	7·6	3·5
Pentose phosphates	15·8	18·0
Unidentified†	61·0	65·3

Conditions: Slices incubated in bicarbonate-saline with 27 mM-K^+ and 10 mM-glucose plus 1·0 μc of [^{14}C]-D-glucose (S. A. 0·34 μc/μmole). Incubation medium volume 2·0 ml; slice weights 250 mg Total activity added was 200,000 counts/min under counting conditions used.

* Calculation from medium content of 22·9 μmoles of glucose.

† Unidentified glycolysis and shunt intermediates plus activity in lipids and proteins extracted from trichloroacetic acid residue during glycogen isolation.

Such studies have been supplemented by experiments in which cat cerebral cortex slices were incubated with [^{14}C]-glucose. Using either [1-^{14}C]-glucose or [6-^{14}C]-glucose as labelled substrate, both $^{14}CO_2$ and [^{14}C]-lactic acid activities derived from each were compared, expressing results as the ratio of activity derived from 6-labelled glucose to that from 1-labelled glucose. The [6-^{14}C]/[1-^{14}C] ratios for $^{14}CO_2$ were 0·99 and for [^{14}C]-lactate were 1·03 from both control slices and those incubated with 10 mM-2-deoxyglucose. Ratios substantially less than 1·0 for $^{14}CO_2$ would be compatible with a

considerable fraction of glucose being metabolized via glucose-6-phosphate dehydrogenase and the oxidative shunt. Ratios greater than 1·0 for [^{14}C]-lactate might be similarly interpreted, although other possibilities would require consideration in this case. The findings indicate little, if any, metabolism of glucose by this route in control slices, and no 'activation' of this pathway in the presence of 2-deoxyglucose. These data for $^{14}CO_2$ from control slices agree with unpublished findings by AGRANOFF and BRADY (1958) on rat cerebral cortex slices, where the [6-^{14}C]/[1-^{14}C] ratio was ~1·0. Previously AGRANOFF *et al.*, (1954) found the ratio for rat diaphragm and kidney also to be ~1·0, but for liver it was ~0·3 under their conditions. In view of the fact that triphosphopyridine nucleotide (TPN) levels are relatively low in brain (KAPLAN, 1958), it is not unexpected to find that metabolism of glucose by glucose-6-phosphate dehydrogenase is not active in cerebral cortex compared to other tissues such as liver.

When such slices were incubated with [^{14}C]-glucose, results summarized in Table 4 were obtained. The presence of a large fraction of the gross amount utilized as 'free', unmetabolized glucose in the tissues (Table 3) is confirmed for those slices incubated with 2-deoxyglucose. This fraction averaged 46 per cent of the gross amount utilized. After correcting for this fraction, the net glucose metabolized by 2-deoxyglucose-inhibited slices amounted to only about one-third of that by control slices. The distribution of labelling from the metabolized [^{14}C]-glucose follows the same general pattern in both controls and slices incubated with 2-deoxyglucose. Deviations of the latter from control patterns of distribution are indicated in Table 5, where results for slices incubated with 2-deoxyglucose are expressed as a percentage of the activity in control fractions.

TABLE 5—PERCENTAGE OF METABOLIZED GLUCOSE IN CONTROL SLICES FOUND IN SLICES INCUBATED WITH 10 mM-2-DEOXYGLUCOSE

	% of controls	
Total glucose metabolized		32·7
Fracsions of total metabolized		
Lactic acid		27·2
CO_2		47·2
Free amino acids		32·0
Glutamic acid	34·6	
Glutamine	23·7	
γ-Aminobutyric acid	27·1	
Aspartic acid	26·4	
α-Alanine	63·0	
Other components		75·5
Glycogen	97·4	
Glucose-6-P	42·0	
Fructose-6-P	35·0	
Pentose phosphates	88·1	

In the inhibited slices, proportionately more of the glucose metabolized was recovered as CO_2 than in control slices, despite the marked decrease in metabolism to lactate and to amino acids associated with the tricarboxylic acid cycle. Metabolism to

α-alanine stands in sharp contrast to the other amino acids. Little, if any, reduction in metabolism of glucose to glycogen or non-nucleotide pentoses occurred, but fructose-6-phosphate shared in the reduction seen for lactate and glutamate.

Interpretation of these data must necessarily be cautious. The distribution of metabolized glucose, as indicated by ^{14}C-activity in various fractions, in control slices is very similar to that reported by BELOFF-CHAIN *et al.*, (1955) for rat cerebral cortex slices under similar conditions. Analogous studies using ^{14}C-labelled pyruvate have given a similar distribution of labelling in glutamic acid, glutamine, γ-aminobutyric acid, and aspartic acid of incubated cat cortex slices (TOWER, 1958*a*) and of incubated rat cortex slices or rat brain *in vivo* (BUSCH, 1955; BUSCH, GOLDBERG and ANDERSON, 1956). In studies on the perfused cat brain *in situ*, GEIGER (1958) has found that a substantial portion of the glucose metabolized does not go directly to CO_2 but is used to resynthesize non-carbohydrate components, such as free amino acids, which are being oxidized simultaneously with glucose. It may be inferred that some of the differences shown in Table 5 represent suppression of these diversionary pathways, as the 2-deoxyglucose-inhibited slices attempt to maintain basic energy-producing metabolism, and that less metabolized glucose can be spared to replete lactate or glutamate pools.

On the other hand in both control slices and those incubated with 2-deoxyglucose, a small but definite percentage is metabolized to glycogen and to free pentoses and these fractions appear little affected by the inhibitory process. Such data may be compatible with the block in conversion of glucose-6-phosphate to fructose-6-phosphate proposed by WICK *et al.* (1957). The detection of ^{14}C-labelling in pentose-phosphates is indicative of operation of the oxidative shunt pathway, but the small percentage of the total metabolized which is recovered in this fraction again suggests that this is not a major pathway for glucose metabolism in cerebral cortex.

Mechanisms of 2-deoxyglucose inhibition

The foregoing evidence provides some support for the mechanism of inhibition suggested by WICK *et al.* (1957), that 2-deoxyglucose-6-phosphate competitively blocks glycolysis at the first stage in this pathway. Recent studies by BLASS and ASHMORE (1958) with rat brain homogenates inhibited by 2-deoxyglucose have demonstrated relief of inhibition by addition of glucose-6-phosphate or fructose-6-phosphate. They found only the latter to be effective in the presence of inhibition by 2-deoxyglucose-6-phosphate. However, the data discussed above suggest that this may not be the sole mechanism nor perhaps even the principal mechanism of inhibition in slices. Despite the reduction in metabolized glucose recovered in fructose-6-phosphate, lactic acid and glutamate (Table 5), there is proportionately more recovered in α-alanine and as CO_2 than in control slices. And evidence cited makes it unlikely that the relatively greater amount of glucose-derived CO_2 arose from the oxidative shunt rather than from the tricarboxylic acid cycle.

WICK *et al.* (1957) also suggested secondary inhibition of hexokinase as an additional possible mechanism. Such an inhibition could arise either from non-competetive inhibition by accumulated glucose-6-phosphate or by depletion of available ATP or both. WOODWARD and HUDSON (1954) suggested the latter possibility also, and SOLS and CRANE (1954) emphasized in a different context the ability of 2-deoxyglucose to isolate the hexokinase reaction.

These possibilities have been re-examined. The behaviour of components of the acid-soluble phosphate fraction of incubated cortical slices under conditions employed here is summarized in Table 6. Control slices exibited levels of inorganic phosphate,

TABLE 6—ACID-SOLUBLE PHOSPHATES IN INCUBATED SLICES
(μmoles/g)

	Initial	60 min	60 min + 2-DG (10 mM)
Inorganic P (6)	5·9 (±0·7)	6·45 (±0·55)	4·3 (±0·35)
Creatine P (5)	1·0 (±0·2)	1·05 (±0·5)	0·5 (±0·2)
Adenosine poly-P (5)	1·85 (±1·1)	1·3 (±1·1)	(<0·1)
Other labile P (5)			2·75 (±0·4)

Numbers of determinations and standard deviations given in parentheses.

creatine phosphate and adenosine polyphosphates comparable to those previously reported, as reviewed by HEALD (1956), making allowance for the effects of 27 mM-potassium in the incubation medium. In the slices incubated with 10 mM-2-deoxyglucose there was a significant decrease in creatine phosphate and extremely low levels of adenosine polyphosphates were obtained. The latter were so low that the methods employed for their isolation and estimation may be subject to some error. Futher studies on this point should probably be done with micro-enzymic procedures.

Two other significant differences appeared in the slices incubated with 2-deoxyglucose. Instead of the rise in inorganic phosphate which regularly occurs in normal slices incubated in presence of 27 mM-potassium, these slices showed a significant decrease together with the appearance of a new labile phosphate constituent not present in control slices. The latter could be readily distinguished from adenosine polyphosphates since it is isolated in the barium-soluble, alcohol-insoluble fraction. From a phosphate balance standpoint, the data in Table 6 seems consistent with diversion of most of the phosphate not found in other constituents of the total fraction to this new component.

On chromatography of hexose and pentose phosphates present in the acid-soluble fraction of incubated slices, findings depicted in Fig. 2 were obtained. Glucose-6-phosphate could not be detected with certainty, but fructose-6-phosphate, a pentose-phosphate and inorganic phosphate were clearly demonstrated in these slice extracts. In addition slices incubated with 2-deoxyglucose showed an extra component occupying the same chromatographic position as authentic 2-deoxyglucose-6-phosphate, and there was obvious reduction in the size of the inorganic phosphate spot consistent with findings in Table 6. Semi-quantitative estimation of the quantity present as 2-deoxyglucose-6-phosphate were of the order of 2–3 μmoles/g, again consistent with data in Table 6. Both SOLS and CRANE (1954) and WICK *et al.* (1957) reported that 2-deoxyglucose-6-phosphate is not further metabolized and that it could be isolated from their tissue preparations. Thus the findings in Table 6 and Fig. 2 are compatible with the interpretation that the new, labile phosphate component in slices incubated with 2-deoxyglucose is 2-deoxyglucose-6-phosphate. An authentic sample of the latter proved to be labile to hydrolytic conditions employed as shown by

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inorganic phosphate released and free 2-deoxyglucose recovered. Thus, it would appear that slices of cortex incubated with 10 mM-2-deoxyglucose contained after 1 hr about 2·75 μmoles/g of 2-deoxyglucose-6-phosphate, that this compound is more labile than other hexose phosphates, and that its formation could account for most of

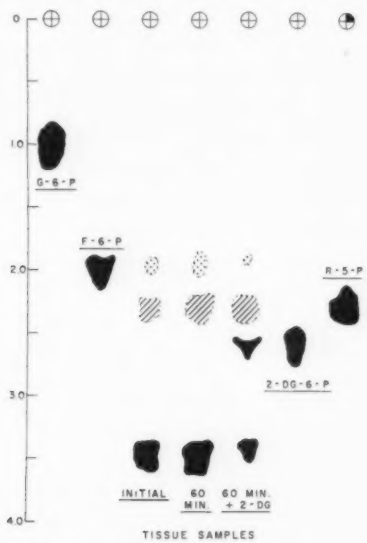

FIG. 2. Reproduction (tracing) of a typical single-dimensional descending paper chromatogram of phosphorylated carbohydrate intermediates isolated from incubated slices of cat cerbral cortex together with authentic standards (0·1 μmole each). Aliquots (50 μl) of slice extracts, equivalent to approximately 30 mg of original tissue, and standards were applied at the top and developed in *n*-amyl acetate–acetic acid–water for 36 hr. Duplicate chromatograms were sprayed with $FeCl_3$-sulphosalicylic acid for phosphate and with *p*-anisidine-HCl for sugars respectively, and the spots visualized have been combined on this tracing. Standards have been referred to glucose-6-phosphate (G-6-P) = 1·0 on the accompanying scale. The position of other standards and their colour reaction with *p*-anisidine–HCl are as follows: fructose-6-phosphate (F-6-P) $\simeq$ 2·0 [yellow-brown]; ribose-5-phosphate (R-5-P) $\simeq$ 2·4 [red]; 2-deoxy-glucose-6-phosphate (2DG-6-P) $\simeq$ 2·7 [brown]; inorganic phosphate $\simeq$ 3·5 [no colour]. Similar values have been obtained by ASHWELL (1957). Definitely detectable compounds isolated from slices are depicted in the centre of the figure: Initial = slices immersed in medium but not incubated ; 60 min = control slices incubated 1 hr; 60 min + 2-DG = slices incubated 1 hr with 10 mM-2-deoxyglucose. The relative prominence of the spots detected is indicated by the degree of shading (stippled—least prominent; solid—most prominent). Spots for slice extracts shown at positions corresponding to standards gave positive reactions for phosphate and colour reactions with *p*-anisidine–HCl similar to those of the respective standards. The spots for slices at position 3·5 gave a reaction for phosphate only and presumably represent inorganic phosphate. Glucose-6-phosphate could not be detected with certainty in slice extracts but was found on radioautograms of chromatographed extracts of slices incubated with ^{14}C-labelled glucose. The latter also showed activity, corresponding to positions in this figure, for fructose-6-phosphate and ribose-5-phosphate (Table 4). Identity of the pentose-phosphate isolated from slices is not established since pentose-phosphates other than ribose-5-phosphate may occupy the same relative position and give the same colour reaction with *p*-anisidine–HCl.

the depletion of energy-rich and inorganic phosphates found in these slices. This concentration is also of the same order as the balance unaccounted for in the analyses of 2-deoxyglucose uptake by these slices (Tables 2 and 3).

On the basis of these findings, a primary effect of 2-deoxyglucose appears to be production of a progressive failure of hexokinase owing to depletion of available ATP.

This is not likely to be a simple phenomenon. ATP is considered to be synthesized in mitochondria and released in a regulated fashion to other cellular loci such as the glycolytic system (see e.g. SIEKEVITZ and POTTER, 1955). Failure of the latter might shift such regulatory systems in favour of retention for tricarboxylic acid support with non-carbohydrate substrates. The failure of glycolysis may of itself deplete available ATP, in addition to any sequestration of phosphate by unmetabolizable 2-deoxyglucose-6-phosphate.

The possibility of overcoming inhibition of glycolysis in slices incubated with 2-deoxyglucose by addition of either glucose-6-phosphate or ATP was investigated. Such additions to slices incubated aerobically were without effect, presumably owing to failure of these compounds to enter slices in sufficient concentration. Under conditions where cellular membrane integrity is less well maintained effects of such compounds on slice metabolism have previously been observed (STERN *et al.*, 1949; TOWER, 1956). Accordingly, these experiments were repeated under anaerobic conditions with results shown in Table 7 and Fig. 3.

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TABLE 7—EFFECT OF 3 MM-SODIUM ATP OR 5 MM-GLUCOSE-6-PHOSPHATE ON INHIBITION OF ANAEROBIC GLYCOLYSIS BY 1 MM-2-DEOXYGLUCOSE IN INCUBATED SLICES (3 MM-K^+)
(μmoles/g)

	0 to 30 min	0 to 60 min	Δ 30 to 60
Control (±ATP or G-6-P) (5)	30·2 (±1·3)	47·5 (±2·0)	+17·3
1 MM-2-Deoxyglucose (8)	18·7 (±1·1)	22·3 (±1·7)	+3·6
+ATP or G-6-P (4) (added at 30 min)		30·7 (±1·1)	+12·0

(30 min values determined manometrically; 60 min values determined both chemically and manometrically.)
Numbers of determinations and standard deviations given in parentheses.

The progressive nature of inhibition of glycolysis by 2-deoxyglucose is well illustrated in Fig. 3. Despite the complication of oxygen uptake, very similar observations were made for slices incubated aerobically, with the slope of the curve being even steeper than that observed anaerobically. When either ATP or glucose-6-phosphate was added after inhibition was already well established, a significant increase in anaerobic glycolysis (determined both manometrically and by direct lactic acid analyses) occurred in supplemented slices compared to controls. Under the same conditions no effects of such additions on control slices were found. As indicated in Table 7, the actions of ATP and glucose-6-phosphate were indistinguishable. A typical experiment using glucose-6-phosphate is shown in Fig. 3.

These investigations on the mechanism of action of 2-deoxyglucose do not permit unqualified delineation of the mechanisms. It is possible that the results in Table 7 and Fig. 3 represent effects of ribose-phosphate derived from adenosine or of fructose-phosphate, often a contaminant of glucose-phosphate preparations, although these

possibilities are considered unlikely. It does seem justifiable to conclude that the metabolic area involved is confined to the hexokinase–glucose-6-phosphate–fructose-6-phosphate steps and that one principal characteristic is failure of hexokinase owing to depletion of available ATP. Relief of inhibition, such as that illustrated in Table 7, is likely to be only temporary. ATP generated by additional glycolysis or that

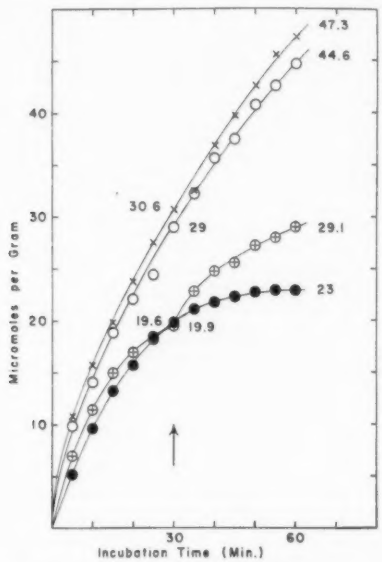

FIG. 3. The effect of addition of 5 mM-glucose-6-phosphate on anaerobic glycolysis of incubated slices of cat cerebral cortex. Incubation medium was bicarbonate–saline–glucose with 3 mM-K^+; gas phase 95% N_2–5% CO_2. Glycolysis is expressed in μmoles/g (ordinate). Control slices are plotted with (○) or without (×) added glucose-6-phosphate. Slices incubated with 1 mM-2-deoxyglucose are plotted as control (●) and those to which glucose-6-phosphate was added (⊕). In both cases the addition was made at 30 min incubation time (arrow), with the 0–30 min manometric glycolysis values given adjacent to the appropriate curve. Final (0–60 min) manometric values were confiirmed by direct estimation of lactic acid content of incubation media. The time course of inhibition of glycolysis by 2-deoxyglucose is well illustrated by the unsupplemented sample (●) compared to its normal control (×).

provided exogenously is competed for by glucose and 2-deoxyglucose at the hexokinase level. Analysis of slices with added ATP showed increased uptake (50 per cent) of 2-deoxyglucose after the addition, compared to unsupplemented controls.

Effects of 2-deoxyglucose inhibition on other systems

A number of metabolic systems in cerebral cortex have been presumed to depend upon glucose metabolism for their maintenance and proper functioning. Factors concerned with metabolism of glutamic acid have been of interest to us. Effects of incubating cat cerebral cortex slices with 10 mM-2-deoxyglucose on glutamate are summarized in Table 8.

Control slices exhibit changes similar to those reported for many previous experiments on normal cortex (TOWER, 1958*a*, *b*). In the presence of 2-deoxyglucose the normal rise of glutamic acid not only fails to occur but a profound depletion is encountered. In spite of this glutamine levels rise significantly, although the increase

is only about 60 per cent of that in control slices. Such an increase was unexpected in view of the poor maintenance of ATP, a necessary co-factor for glutamine synthesis. The characteristics of glutamate metabolism in these slices make it unlikely that the extra glutamine was derived from protein breakdown (VRBA, 1956) since glutamine so released is rapidly converted to glutamic acid and free ammonia. Normally incubation slices lose some glutamate (mostly as glutamine) to the incubation medium prior to initiation of optimal incubation conditions, but during incubation much of this is taken back by the slices, as shown in Table 8, a process which is energy-dependent

TABLE 8—EFFECT OF 10 mM-2-DEOXYGLUCOSE ON GLUTAMATE METABOLISM IN INCUBATED SLICES (27 mM-K^+)
(μmoles/g)

	Glutamic acid		Glutamine		Medium glutamate	
	Level	Change	Level	Change	Level	Change
Control (2)						
Initial	7·55		4·3		4·3	
60 min	9·95	+2·4 (±0)	6·75	+2·45 (±0·2)	3·3	−1·0 (±0·3)
+2-Deoxyglucose (2)						
60 min	2·8	−4·75 (±0·25)	5·8	+1·5 (±0)	4·4	+0·1 (±0·07)

(Respective tissue ammonia levels were: 2·5; 2·5; and 2·8)
Numbers of determinations and standard deviations given in parentheses.

(STERN *et al.*, 1949; TOWER, 1958*a*). In the presence of 2-deoxyglucose this process fails, and it may be more than coincidental that the fraction not reabsorbed by these slices would account for the lower glutamine levels in these slices.

Various types of evidence strongly implicate metabolized glucose as the major source of free glutamic acid in cerebral cortex and of free glutamine synthesized from it (TOWER, 1958*a*, *b*). Data in Table 4 form part of such evidence. Under adverse metabolic conditions glutamine appears to be much more stable than glutamic acid (TOWER, 1958*b*). The effects of 2-deoxyglucose here are expressed primarily in impairment of glutamic acid metabolism. With identical experimental conditions where slices are incubated under partial hypoxia, similar but less severe changes in glutamic acid have been observed, but in addition glutamine synthesis fails significantly and glutamate continues to 'leak' from slices to medium during incubation (TOWER, 1958*a*, *b*). The contrast between such findings and those in Table 8 suggest that maintenance of free glutamic acid may be more closely related to adequate glucose metabolism whereas glutamine may depend upon energy production regardless of its source.

Analogous studies on electrolyte metabolism are summarized in Tables 9 and 10. Prior to initiation of optimal incubation conditions slice potassium is normally lost to the mediun and replaced by an equivalent of sodium (TERNER *et al.*, 1950; PAPPIUS and ELLIOTT, 1956; TOWER, 1958*a*). This situation is illustrated by the initial slice levels shown in Table 9. During incubation slices normally take back the lost

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potassium and expel excess sodium to achieve an electrolyte constitution by the end of incubation close to that found on cortical biopsy. In the presence of 10 mM-2-deoxyglucose this process is significantly impaired, as shown in Table 9. At the same time

TABLE 9—EFFECT OF 10 mM-2-DEOXYGLUCOSE ON ELECTROLYTE METABOLISM IN INCUBATED SLICES (27 mM-K^+) (μEq./g)

	Potassium		Sodium		Chloride	
	Level	Change	Level	Change	Level	Change
Control (2)						
Initial	45·8 (±1·8)		100·0 (±11·3)		59·2 (±0·4)	
60 min	91·8 (±6·7)	+46	59·2 (± 4·6)	−40·8	44·8 (±1·7)	−15·4
+ 2-Deoxyglucose (2)						
60 min	61·8 (±10·3)	+16	83·8 (± 1·8)	−16·2	59·0 (±5·6)	−0·2

(Expected tissue levels on biopsy: K^+ 95·6; Na^+ 57; Cl^- 38. On incubation comparable changes detected in incubation medium).

Numbers of determinations and standard deviations given in parentheses.

the normal restitution of fluid spaces also fails in such slices (Table 10). The data in Table 10 indicate that these electrolyte changes are real and not attributable to mere shifts in fluid spaces. It is of interest that the swelling of slices incubated with 2-deoxyglucose is less than that of control slices. Such swelling occurs only in the chloride

TABLE 10—TISSUE SPACES IN INCUBATED SLICES (27 mM-K^+)

	Non-chloride space		Chloride space		Assumed solids
	%	Change	%	Change	%
Control (2)					
Initial	37·5 (±0·7)		46·5 (±0·7)		16
60 min	48·5 (±0·5)	+11	35·5 (±0·5)	−11	16
+ 2-Deoxyglucose (2)					
60 min	37·5 (±3·5)	0	46·5 (±3·5)	0	16

SWELLING OF INCUBATED SLICES (27 mM-K^+)
Percentage of initial weight (4)

Initial	60 min	60 min + 10 mM 2-deoxyglucose
30·3 (±3·0)	65·7 (±3·0)	55·3 (±3·8)

Numbers of determinations and standard deviations given in parentheses.

space (PAPPIUS and ELLIOTT, 1956; TOWER, 1958*a*) and has been corrected for in data given in Table 9.

Comparison of these results with those for slices incubated under partial hypoxia (TOWER, 1958*a*) indicates that the latter are more severely impaired. Potassium levels were lower, sodium levels were higher, no significant changes occurred on incubation, and swelling was much more extensive. This comparison suggests that the maintenance of normal electrolyte composition may be more dependent upon energy production regardless of substrate than upon adequate glucose metabolism *per se*.

Since the action of 2-deoxyglucose appears to involve both an inhibition of glucose utilization and a reduction in available energy-rich compounds, it is difficult to distinguish the relative importance of these two factors on glutamate and electrolyte metabolism. This situation illustrates the necessity of understanding the mechanism of action of a metabolic inhibitor and the problems in interpreting its effects even when such mechanisms are reasonably evident.

Cerebral glucose metabolism

In studies on the metabolism of the perfused cat brain *in situ*, GEIGER (1958) has reported that non-carbohydrate substrates are oxidized simultaneously with metabolized glucose and that a substantial fraction of the latter is used for resynthesis of these oxidized non-carbohydrate constituents. Thus only about one-third of metabolized glucose appears promptly as CO_2 and another third as free amino acids, the remainder going to lipids, proteins and the like. These findings do not contradict the classical concept that glucose is normally the only substrate oxidized by the brain, but merely restate it in modified form. It is clear that the process of glucose oxidation is partially indirect via non-carbohydrate compounds to which it has been diverted and which in turn are partly oxidized. It may be inferred that eventually most of the glucose metabolized by the brain will be recovered as CO_2. In the absence of adequate glucose supplies, non-glucose substrates are oxidized, but dysfunction ensues because insufficient glucose is available to replace them, as studies by GEIGER (1958) clearly show.

On this basis over 80 per cent of the oxygen consumption found for control slices (Table 1) can be accounted for by CO_2 and amino acids derived from glucose (Table 4). In contrast, for slices incubated with 10 mM-2-deoxyglucose, only about 40 per cent of the oxygen consumption can be similarly accounted for, and the lower RQ of these slices (0·8) is consistent with this calculation. From the results of glutamate and electrolyte studies (Tables 8, 9 and 10) it is obvious that the ability of endogenous substrates to support metabolic functions and yield energy is inadequate when the metabolism of glucose is significantly interfered with. Recent emphasis on findings that stimulated sympathetic ganglia or cortical slices stimulated during incubation do not use the extra glucose consumed for direct support of increased oxygen consumption but for lactic acid production (HOROWICZ and LARRABEE, 1958) must be tempered by the foregoing considerations. The production of lactic acid by neural tissue preparations *in vitro* is somewhat anomalous, since most of it diffuses out of the tissue and becomes less readily available as a substrate for cellular metabolism. In a sense much of this is 'wasted' substrate, which *in vivo* might be more efficiently utilized or regulated (GEIGER, 1958).

As the metabolic organization of neural tissues is investigated in more detail, the more apparent it becomes that neurones have evolved a basic energy-producing system

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which is designed to cope with many levels of activity and substrate demand and to insert regulatory actions when and where they may be appropriate. The ability to call upon non-carbohydrate substrates at times of intense activity and then replace them subsequently from metabolized glucose without disruption of vital functions indicates a highly efficient type of organization. The importance which such a concept places upon the supporting systems, such as amino acids, proteins and lipids (GEIGER, 1958; TOWER, 1958*a*; VRBA *et al.*, 1958) necessitates fuller investigation and understanding of these systems.

Regardless of the wealth of data on glucose and oxidative metabolism in neural tissues *in vitro* and the sometimes contradictory interpretations adduced, the fact remains that insufficiency of glucose supplies to the central nervous system *in vivo* rapidly result in irreparable dysfunction. 2-Deoxyglucose is an excellent case in point. *In vivo* doses of 12 m-moles/kg produce seizures and coma (LANDAU and LUBS, 1958), which reflect grave disruption of neuronal metabolism. Similar concentrations *in vitro* produce metabolic defects analogous to those encountered *in vivo*, and the nature of these *in vitro* defects are clearly the basis for *in vivo* dysfunction—the inability of neurones to utilize available glucose in quantities sufficient to support their metabolic requirements. Despite some uncertainties in details, the *in vitro* findings with 2-deoxyglucose discussed in this paper appear to conform satisfactorily to observations *in vivo*. Together they reaffirm the essentiality of adequate glucose metabolism to normal neuronal function and activity.

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SUMMARY

Effects of 2-deoxy-D-glucose on metabolism of incubated slices of cat cerebral cortex have been investigated. Inhibition of aerobic and anaerobic glycolysis and to a lesser extent, of oxygen consumption occurs, the degree being dependent upon concentration. For glycolysis 50 per cent inhibition is produced anaerobically by 0·8 mM-2-deoxyglucose and aerobically by 4 mM. Oxygen uptake is inhibited 25 per cent at about 10 mM-2-deoxyglucose compared to 75 per cent inhibition of aerobic glycolysis at this concentration. The RQ of such slices averages 0·8 compared to 0·92 for controls.

Under these conditions (10 mM-2-deoxyglucose, aerobic) gross uptake of glucose is 60 per cent of control slices with about 45 per cent of this total present as 'free' unmetabolized glucose in the slice. Thus net metabolism of glucose in slices incubated aerobically with 10 mM-2-deoxyglucose is only 33 per cent of control slices, average net consumption being 13·4 μmoles/g per hr and 40·9 μmoles/g per hr respectively.

No accumulation of glucose-6-phosphate or intermediates in any of the pathways alternative to glycolysis can be demonstrated in the presence of 2-deoxyglucose. $^{14}CO_2$ and [^{14}C]-lactic acid derived from [1-^{14}C]-glucose or [6-^{14}C] glucose are of the same order, the [6-^{14}C]/[1-^{14}C] ratios being 0·99 and 1·03 respectively for both control and 2-deoxyglucose-inhibited slices, so that no 'activation' of the oxidative shunt pathway is produced by 2-deoxyglucose inhibition. The fraction of glucose metabolized by this pathway appears to be minor in control slices. Detailed analysis of the distribution of metabolized [^{14}C]-glucose indicates a similar pattern for controls and for slices incubated with 10 mM-2-deoxyglucose. In the latter the percentage of control amounts metabolized to any given component or fraction is about 25–35 per cent, but proportionately more is recovered in CO_2 (47 per cent) and α-alanine (63 per cent) and

little if any reduction in percentage metabolized to glycogen (97 per cent) or to non-necleotide pentose phosphates (88 per cent) is found.

Compared to controls, slices incubated with 10 mM-2-deoxyglucose exhibit marked depletion of creatine phosphate and adenosine polyphosphates together with an accumulation of 2–3 μmoles/g per hr of 2-deoxyglucose-6-phosphate. Although the findings do not rule out a block of glycolysis by the latter at the glucose-6- to fructose-6-phosphate step (as previously proposed by others), they indicate that a primary effect of 2-deoxyglucose is failure of hexokinase owing to depletion of available ATP. Relief of inhibition of anaerobic glycolysis can be achieved by addition of 3 mM-ATP or 5 mM-glucose-6-phosphate to slices already inhibited by 1 mM-2-deoxyglucose.

During incubation with 10 mM-2-deoxyglucose (aerobic) slice levels of glutamic acid are profoundly depleted and uptake of glutamate from the medium fails, whereas glutamine levels are maintained fairly well. In control slices glutamic acid rises 2·4 μmoles/g in 1 hr to a final level of 9·95 μmoles/g, while in 2-deoxyglucose-inhibited slices it decreases 4·75 μmoles/g to a final level of 2·8 μmoles/g in the same period.

The ability of such slices to restore normal electrolyte composition is also markedly impaired. After 1 hr incubation control slices contain 91·8 μEq./g of potassium and 59·2 μEq./g of sodium, compared to 61·8 μEq./g and 83·8 μEq./g respectively in slices incubated with 10 mM-2-deoxyglucose. In the latter restoration of normal tissue fluid spaces, as determined by chloride, also fails to occur.

The data are discussed in relation to the total problem of cerebral glucose metabolism. These *in vitro* effects of 2-deoxyglucose complement previous *in vivo* observations and both re-emphasize the necessity for adequate glucose metabolism to maintain normal neuronal function and activity.

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ANALYSIS OF ARTERIAL AND CEREBRAL VENOUS BLOOD FROM THE RABBIT

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IN reviewing the general metabolism of the brain *in vivo*, KETY (1957) and MCILWAIN (1959) have emphasized that only in glucose and a few related metabolites have appreciable cerebral arteriovenous differences been observed. It was accordingly felt desirable to make systematic exploratory studies of other groups of compounds, and also, as much of the available data refers to man, to use an experimental animal.

The rabbit was chosen for this purpose, as it can afford a reasonable volume of blood, and a method has been devised for sampling cerebral venous blood at the superior sagittal sinus (torcula) in the conscious animal (RODNIGHT and TRESIZE, 1957). The present paper describes findings with respect to glucose, lactic acid, and keto-acids which are in line with those already established in other species, and initial studies of amino acids, indole compounds, ascorbic acid and purines.

EXPERIMENTAL

Blood samples

Adult male non-fasting rabbits weighing 2·5–3·5 kg were used. Immediately before sampling they were given 1500 i.u. of heparin intravenously. Arterial blood was taken by means of a polythene cannula from the posterior auricular artery at the base of the ear. A simultaneous sample of cerebral venous blood was taken from the junction of the superior longitudinal and lateral venous sinuses (torcula). The sampling, and a preliminary operation which permitted blood sampling from conscious rabbits, have been described by RODNIGHT and TRESIZE (1957).

In preliminary experiments blood was caused to drip directly from the cannula into a known weight of the appropriate fixing agent at 4°C. Analytical values from such filtrates were compared with others obtained from the blood which had been allowed to stand at room temperature for 1 hr before precipitation. Marked changes on standing occurred with carbohydrate metabolites and purine constituents, but not with the other constituents analysed; with these latter constituents, therefore, samples were taken through the cannulae to silicon-treated syringes, and from these to haematocrit tubes or fixing agents, within 30 min of withdrawal from the animals. All analyses were of whole blood unless stated otherwise, and the arterial and venous specimens from a given experiment were throughout handled together, in precisely the same way and with the same reagents. Unless otherwise indicated specimens were obtained from conscious rabbits. When anaesthesia was required pentobarbitone sodium (Nembutal, Abbott Laboratories) was given intravenously at 30–40 mg/kg body weight.

Analytical methods

Optical densities in the visible spectrum were determined in a Unicam S.P 600 spectrophotometer; for measurements in the ultra-violet range a Hilger Uvispek instrument was used. Packed cell volumes were determined by centrifuging the blood sample at 2000g for 3 min in a Wintrobe haematocrit tube.

In the analysis of blood for ascorbic acid and 5-hydroxytryptamine, saturation of the blood with carbon monoxide before precipitation of the proteins was found necessary (see below). This was done

by placing the sample in a conical flask of capacity at least ten times the volume of the sample. CO, prepared from HCOOH and H_2SO_4 and washed with 0·1 N-NaOH, was passed through the flask for 15 min whilst the contents were constantly swirled. Excess CO was displaced with N_2 before the sample was pipetted.

Glucose. The arsenomolybdate method of NELSON (1944) modified by KING (1953) was used. The sample was pipetted into the fixing agent within 1 min of withdrawal.

Lactic acid and keto-acids. When blood was shed into ice-cooled tubes these constituents changed within a few minutes. LONG's (1944) method of stabilizing them was therefore adopted: blood (approximately 2·5 ml) was shed in tubes which contained 42 mg citric acid (brought to pH 4 with NaOH and dried), 10·5 mg NaF and 10 mg cetyltrimethylammonium bromide, and were shaken in ice-water.

For lactic acid 1 ml was shaken with 3 ml ice-cold 10% trichloroacetic acid (TAC) and centrifuged after 15 min. Of the supernatant, 0·5 or 1 ml samples were added to 4 ml $CuSO_4.5H_2O$, 2·5%, and the determinations completed according to BARKER and SUMMERSON (1941).

For total keto-acids 1 ml of blood was added to 5 ml of ice-cold 10% (TCA) and centrifuged. The supernatant was filtered and 3 ml taken for determination by the method of FRIEDEMANN and HAUGEN (1943). The standard curve was constructed using pyruvic acid, and the keto-acid results are expressed in terms of this substance.

Amino acids and related compounds. Proteins were precipitated by adding the sample to three times its volume of ethanol. After filtering, the ethanol was removed *in vacuo* and the extract defatted with light petroleum. It was then concentrated by freeze-drying, made up to one-tenth of the volume of blood used with 75% (v/v) ethanol and centrifuged to remove insoluble matter. In some experiments extracts were examined at this stage by paper chromatography; otherwise they were further treated by one of the following procedures. (a) The neutral amino acids were separated by ion-exchange on Amberlite IRH (H-form) and eluted with 2 N-NH_4OH. (b) Basic compounds were absorbed on Zeo-Karb 226 (buffered at pH 5 with triethylamine) and then eluted with N-formic acid. (c) Aromatic compounds were isolated using de-activated charcoal and phenol according to ASATOOR and DALGLEISH (1956).

In all three procedures the eluates were concentrated by freeze-drying and the re-constituted extracts examined by two-dimensional paper chromatography on Whatman No 20 paper. The first solvent was 80% (w/v) phenol containing 0·1% α-benzoin oxime; sometimes ammonia to approx. 0·2M was added. For the second solvent the *n*-butanol-acetic acid mixture of PARTRIDGE (1946) was generally used. Both ascending and descending techniques were employed.

Purine derivatives. Blood (approx. 5 g) was shed into perchloric acid (30% w/v, 3 ml) cooled in ice and analysed within 1 hr of collection. No detectable hydrolysis of nucleotides occurred during 4 hr at 5°C as judged by subsequent analysis for adenine in free form or as nucleotide. The perchlorate mixture was weighed, centrifuged and the supernatant neutralized with 10 N-KOH (bromocresol blue), $KClO_4$ removed by centrifuging and the supernatant made just acid with acetic acid. Purine derivatives were separated by the method of KERR and SERIADARIAN (1945), omitting the precipitation with Cu salts. Nucleotide fractions, precipitated by uranyl acetate, were hydrolysed in 5 ml N-H_2SO_4 at 100° for 1 hr, the uranium precipitated by KOH and the supernatant made up to 10 ml. Portions (2 ml) were taken for precipitation of Ag salts of the purines at pH 4. The various Ag precipitates were heated 1 hr at 100° with 4 ml N-HCl, the mixtures centrifuged and the supernatants dried overnight *in vacuo* with P_2O_5 and NaOH. The residues in N-HCl were applied to Whatman No 1 paper and chromatograms developed in a 2N-HCl/*iso*propanol mixture (WYATT, 1951). In trial experiments, adequate separation by descending paper chromatography of adenine, guanine and hypoxanthine occurred within 48 hr. Spots were located in ultraviolet light (HOLIDAY and JOHNSON, 1949), cut out and eluted in 4 ml N-HCl observing the precautions described by WYATT (1951). Purines were identified by comparison with marker spots and by their absorption peaks and nadir in N-HCl and at pH 10 (WYATT, 1955). Adenine was estimated absorptiometrically at 262 mμ and guanine and hypoxanthine at 248 mμ.

5-Hydroxytryptamine (5-HT) and other indoles. Blood, previously saturated with CO, was added to 4 vols of ice-cold acetone in an all-glass homogenizer and the precipitate ground for 15 sec. The tube was centrifuged, an aliquot of the supernatant taken, the acetone removed *in vacuo* and the aqueous residue defatted with light petroleum. After dilution to a suitable volume the 5-HT content was determined by the rat-uterus preparation of AMIN, CRAWFORD and GADDUM (1954). Treatment

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with CO was necessary for the quantitative recovery of 5-HT from whole blood (RODNIGHT, 1958).

Aliquots of the same acetone extracts were also examined by paper chromatography for indoles. After the defatting stage a volume of the aqueous extract equivalent to 5 ml of blood was concentrated by freeze-drying to exactly 1 ml and 19 vols of acetone added. The solution was filtered and the acetone and water in the filtrate removed *in vacuo*. The dry residue was dissolved in 0·5 ml of 75% (v/v) ethanol and 4·5 ml of acetone were added. After centrifuging the supernatant was concentrated and quantities equivalent to 2 ml of blood examined by two-dimensional chromatography on 9 in. square sheets of Whatman No 20 paper. The chromatograms were run for 18 hr in *n*-butanol–acetic acid–water (PARTRIDGE, 1946) and afterwards for 6 hr in propan-1-ol–water–NH_3 (80 : 15 : 5 v/v). Indoles were detected by dipping the sheets in the *p*-dimethylaminobenzaldehyde reagent of JEPSON (1955).

Creatine. The method of EGGLETON, ELSDEN and GOUGH (1943) was used; sulphydryl compounds which interfere with the reactions were removed by *p*-chloromercuribenzoic acid (ENNOR and STOCKEN, 1953). Proteins were precipitated by adding 2 ml blood to 2 ml water, followed by 2 ml 10% sodium tungstate and 2 ml 0·67 N-H_2SO_4. A standard curve was constructed with every experiment, using standards dissolved in the precipitating solution.

Ascorbic acid. The reduced form was determined by an adaptation of the dye method of MINDLIN and BUTLER (1937), and oxidized forms by the 2 : 4-dinitrophenylhydrazine method of ROE, MILLS, OESTERLING and DAMRON (1948). Proteins were precipitated by the addition of 6 ml blood, saturated with CO, to 18 ml of freshly prepared, ice-cold 3% (w/v) HPO_3 containing 4% TCA; inclusion of the latter was necessary to obtain clear filtrates from whole blood. After centrifuging, the slightly cloudy supernatant was filtered and the filtrate divided into three parts, which were treated as follows:

(a) To 9 ml, 0·22 ml of 2% *p*-chloromercuribenzoic acid in 0·5 N-NaOH was added and after standing for 5 min the mixture was centrifuged (OWEN and IGGO, 1956). A portion (4 ml) of supernatant was transferred to a cuvette with a 3 cm light path and brought to pH 3·5 with 1 ml of 17% sodium acetate. The dye solution used to determine the ascorbic acid contained 0·004% 2 : 6-dichlorophenolindophenol and 4·1% sodium acetate, adjusted to pH 7 with acetic acid; 1 ml was added to the cuvette and after rapid mixing the density was measured at 518 mμ at 30 sec from the time of addition. The final pH of the mixture was 4. To obtain the optical density of the blank, excess ascorbic acid was added and the reading repeated.

Standard curves were constructed from solutions prepared in an appropriate dilution of the HPO_3–TCA solution. STEWART, HORN and ROBSON (1953) found that TCA interfered with the determination of ascorbic acid by the dye method; this observation was not confirmed in our experiments, for after the initial decolorization no further fading occurred. CO treatment was indicated by OWEN and IGGO (1956) and its efficacy confirmed in the present work. In three experiments recoveries of ascorbic acid (5 μg/ml blood) added within 10 min of withdrawal and before saturation with CO, were 90, 93, and 93 per cent, but in one instance where CO treatment was omitted it was only 49 per cent. Inexplicably low recoveries (less than 50 per cent in two experiments) were also obtained using CO-treated blood, when proteins were precipitated by 1·5 parts of 6% (w/v) HPO_3, as recommended by OWEN and IGGO (1956).

(b) This aliquot was used to determine total ascorbic acid (reduced and oxidized forms). To 2·4 ml of filtrate one drop of Br_2 was added to oxidize ascorbic acid and the excess Br_2 removed with a stream of N_2. Then 0·3 ml of 10% thiourea and 0·3 ml of 4% 2 : 4-dinitrophenylhydrazine in 18 N-H_2SO_4 were added and the mixture incubated overnight at 30°. Colours were developed with 3 ml of 85% (v/v) H_2SO_4, added while the solution was cooled in ice-water. Densities were measured at 540 mμ after 30 min in a 3 cm cuvette.

(c) The oxidized forms native to the blood were determined by the above procedure omitting the bromination. Standards of ascorbic acid dissolved in HPO_3–TCA solution were treated in parallel with blood extracts in both (b) and (c).

RESULTS

Sampling technique. In sampling by the present technique from the torcula the risk of contaminating the cerebral venous blood by blood from other sources was negligible. There was however a small risk of contamination from arterial blood by the accidental cutting of an arteriole. For this reason, and also to confirm normal

cerebral metabolism of carbohydrate, the arteriovenous difference in glucose was determined as a routine in all experiments. In forty-seven determinations on twenty-six animals, the mean arterial and cerebral venous glucose concentrations were 115·2 and 102·0 mg/100 ml (mg per cent) respectively, giving a mean arteriovenous difference of 13·2 ± 3·2 mg (standard deviation). In one instance only, a difference of 2 mg per cent was observed; this result is not included in the above figures, nor were the analytical results from the specimens accepted.

TABLE 1.—GLUCOSE, LACTIC ACID AND KETO-ACIDS OF RABBIT BLOOD

For lactic acids and keto-acids arterial blood from the auricular artery and cerebral venous blood from the superior sagittal sinus were shed to ice-cooled tubes containing citrate, fluoride and cetavlon, and determinations completed as described in the experimental section. Glucose was determined on 0·1 ml samples collected immediately afterwards. Values are the mean of closely agreeing duplicates. Results for keto-acids are expressed in terms of pyruvic acid.

Animal specimen	Glucose (μmoles/ml)			Lactic acid (μmoles/ml)			Keto-acids (μmoles/ml)		
	Arterial	Venous	V–A	Arterial	Venous	V–A	Arterial	Venous	V–A
85 (19.11.57)	4·55	3·55	−1·00	—	—	—	0·528	0·534	0·006
82 (28.11.57)	5·44	4·66	−0·78	0·45	0·56	0·11	0·111	0·116	0·005
84 (3.12.57)	7·48	7·10	−0·74	1·89	2·25	0·36	0·284	0·256	−0·028
84 (10.12.57)	3·60	3·10	−0·50	two conflicting values: conclude no certain change			0·195	0·218	0·023
82 (12.12.57)	5·84	5·00	−0·84	1·10	1·30	0·20	0·272	0·250	−0·022
86 (1. 1.58)	6·44	5·40	−1·04	1·66	1·66	0	0·318	0·341	0·023
87 (7. 1.58)	6·72	5·34	−1·38	0·94	1·05	0·11	—	—	—
Means	5·78	4·88	−0·90	1·21	1·36	0·15	0·285	0·286	0·001

Packed cell volumes were also determined in the majority of experiments. The mean value for arterial blood was 33·97 ± 4·56 per cent and the mean difference between arterial and venous specimens was 0·37 ± 0·097 per cent (S.D., thirty-eight specimens).

Glucose, lactic acid and keto-acids. The arteriovenous differences in glucose of 13·2 mg per cent quoted above is close to the value of 13·1 mg per cent observed in anaesthetized dogs by HIMWICH and FAZEKAS (1937), but it is some 20 per cent higher than the upper limit of the range (9–11 mg per cent) established in human subjects by several authors KETY, 1957).

Results for lactic acid and keto-acids as well as some individual glucose determinations (expressed in μmoles/ml) are given in Table 1. The small arteriovenous difference in lactic acid of 0·16 μmoles/ml is of the same order as that of 0·18 μmoles/ml found by GIBBS, LENNOX, NIMS and GIBBS (1942) in man. KETY (1957), however, was unable to demonstrate any release of lactate by the brain in man. In total keto-acids the present results show on the average no arteriovenous difference, which agrees with the observations of KETY (1957), but not with those of HIMWICH and HIMWICH (1946), who found in man a small cerebral production of pyruvic acid of 0·025 μmoles/ml.

Amino acids. Blood samples from anaesthetized and from conscious animals were examined by one or all of the methods described. Using extracts which had not been submitted to any of the ion-exchange or charcoal absorption procedures, satisfactory chromatograms from volumes equivalent to 0·06 ml of blood were obtained. With purified extracts from 0·2 to 2 ml could be examined, depending upon the desalting procedure used. The amino acids were detected by dipping the sheets in 0·2 per cent ninhydrin in acetone and heating for 3 min at 80°. In most cases identification was by comparison of R_F values with standards, but where other tests, such as EHRLICH's reaction for indoles, were available these were always used. The developed chromatograms were examined immediately and after 24 hr, and the pattern and intensity of the spots compared.

For most amino acids the accuracy of determinations made by visual comparison is around ± 20 per cent and within this limit no quantitative arteriovenous differences were found; nor were qualitative differences in the pattern of the spots, including several unidentified spots, detected. Samples from anaesthetized and conscious animals also gave identical results. Typical whole blood amino acid levels for rabbits determined during these experiments are given in Table 2, where they are compared with value for rabbit serum quoted by DUBREUIL and TIMIRAS (1953). Considering that the present results refer to whole blood, there is reasonably good agreement between the two sets of figures.

Purine derivatives. As noted above, blood for analysis for purines was shed immediately into perchloric acid. In preliminary experiments in which this was not done, the free adenine of rabbit blood was found to increase from 3 μg/ml 5 min after shedding, to 4·5 μg/ml 20 min after shedding. Corresponding values for free hypoxanthine were 0·5 and 1·3 μg/ml. In blood shed into perchloric acid, hypoxanthine was not detected while adenine was at the lower level quoted in Table 3, and nucleosides were not detected by the methods employed, implying that less than 0·5 μg of adenine/ml was present in this form.

The nucleotide fraction from blood shed into perchloric acid contained guanine and adenine (Table 3) at concentrations similar to those found by CONWAY and COOK (1939); no arteriovenous difference was found. The free purine fraction always contained adenine, and a smaller amount of guanine which could not be evaluated by the methods used. The level of adenine was greater in anaesthetized than in normal animals whether pentobarbitone or ether was used as the anaesthetic. During anaesthesia with pentobarbitone, cerebral venous blood contained about 0·4 μg/ml less adenine than arterial blood: a significant difference with a probability $P = 0{\cdot}05$. No arteriovenous difference was found in conscious animals.

5-Hydroxytryptamine and other indoles. Arterial and cerebral venous whole blood 5-hydroxytryptamine was determined in three conscious animals by bioassay on the rat uterus with an accuracy of $\pm 5\%$; the arterial and venous concentrations in μg/ml blood were as follows: 5·4, 5·2; 5·0, 4·9; 5·1, 5·4. Thus no arteriovenous difference in 5-hydroxytryptamine could be demonstrated by this method. The present experiments give an average of 5·2 μg/5-hydroxytryptamine per ml rabbit whole blood; the value reported by SHORE, PLETSCHER, TOMICH, KUNTZMAN and BRODIE (1956) was 3·5 μg/ml rabbit blood.

Chromatograms of acetone extracts of rabbit blood examined with the Ehrlich reagent of JEPSON (1955) showed three purple spots. The major spot corresponded in

TABLE 2.—AMINO ACIDS IN RABBIT WHOLE BLOOD

Values quoted for the present investigation were obtained by visual comparison of spots with standards on paper chromatograms and are accurate to about ±20 per cent. For most estimations venous blood was used; no arteriovenous differences were observed.

Amino acid	Values of DUBREUIL and TIMIRAS (1953) (μg/ml serum)	Present values (μg/ml blood)
Alanine	17	13
β-Alanine and citrulline	—	11
γ-Aminobutyric acid	—	trace
Arginine	19	30
Aspartic acid	<3	8
Cystine	—	2
Glutamic acid	9	8
Glutamine	43	30
Glycine	52	25
Histidine	—	<5
Leucine	25	15
Lysine	8	15
Methionine	6	5
Phenylalanine	—	20
Proline	15	24
Serine	6	4
Threonine	9	10
Tryptophan	—	12
Tyrosine	<8	4
Valine	15	18

TABLE 3.—PURINES OF RABBIT BLOOD

Arterial and cerebral venous specimens (number in parenthesse, followed by S.E.M. of the result) were shed simultaneously into ice-cold perchloric acid.

Condition of animal	Whole blood levels (μg/ml)				
	Category determined	Arterial		Cerebral venous	
		Adenine	Guanine	Adenine	Guanine
Anaesthetized (pentobarbitone)	Free	1·54 (8) ±0·28	—	1·14 (8) ±0·04	—
Anaesthetized (pentobarbitone)	Nucleotide	86·2 (7) ±13·9	14·1 (5) ±7·3	89·4 (7) ±13·8	16·9 (4) ±8·0
Conscious	Free	0·6	—	0·65 (4)	—

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TABLE 4—ASCORBIC ACID IN RABBIT BLOOD

Arterial and cerebral venous specimens (number in parentheses followed by the S.E.M. of the result) were withdrawn simultaneously with siliconed syringes.

Condition of animal	Specimen analysed	Whole blood levels						Arteriovenous difference		
		Arterial			Cerebral venous					
		Ascorbic acid	Oxidized ascorbic acid	Total ascorbic acid	Ascorbic acid	Oxidized ascorbic acid	Total ascorbic acid	Ascorbic acid	Oxidized ascorbic acid	Total ascorbic acid
Anaesthetized	Plasma	2·64 (8) ±0·36	—	5·94 (4) ±0·56	2·41 (8) ±0·40	—	5·85 (4) ±0·73	0·23	—	0·09
Anaesthetized	Whole blood	6·75 (4) ±1·36	—	8·41 (4) ±1·07	6·97 (4) ±1·26	—	8·71 (4) ±0·99	−0·22	—	−0·30
Conscious	Whole blood	4·96 (8) ±0·46	1·80 (8) ±0·28	6·88 (8) ±0·51	4·70 (8) ±0·44	1·81 (8) ±0·28	6·82 (8) ±0·49	0·26*	−0·01	0·06

* $P < 0·05$. Other differences were not significant

R_F to 5-hydroxytryptamine. A smaller spot of tryptophan was also seen, representing however only part of the tryptophan in the blood owing to its relative insolubility in acetone. The third spot had the same properties as an unknown compound often noticed in acetone extracts of human blood (RODNIGHT, unpublished observation). It gave a reddish purple colour with the Ehrlich reagent, developing to a maximum in 15 min and slowly fading to grey in about 6 hr. Its R_F in *n*-butanol–acetic acid–water was 0·60, in propan-1-ol–water–NH_3, 0·30. No arteriovenous difference in the pattern or intensity of the indole spots was noted. Tryptamine, or 5-hydroxyindole-acetic acid could not be detected, although both compounds were quantitatively recovered from blood by the acetone extraction method.

Creatine. Values for creatine in rabbit arterial blood averaged 1·98 $\pm$ 0·16 (S.E.M.) mg/ml in five anaesthetized and two conscious animals. The mean arteriovenous difference was 0·0025 $\pm$ 0·017 (S.E.M.) mg/100 ml, which was not significant.

Ascorbic acid. In early experiments on animals anaesthetized with pentobarbitone reduced ascorbic and total ascorbic acid were estimated in plasma, separated within 10 min of withdrawal. No significant arteriovenous difference was found in this series of eight experiments (Table 4). The mean of the reduced ascorbic acid determination is close to that of 2·6 μg/ml reported by LANGHAM and HEALD (1956). Analysis of whole blood from anaesthetized animals failed to show any consistent change.

A group of eight conscious animals was used to study arteriovenous differences across the brain of reduced, oxidized and total ascorbic acid. Here a small difference of 0·26 μg, significant to $P < 0·05$, was apparently observed in reduced ascorbic acid. Oxidized ascorbic acid and total ascorbic acid, however, remained unchanged making it unlikely that the observed change was an oxidation of the ascorbic acid at the brain. As described in the Experimental section reduced ascorbic acid was estimated by reduction of 2 : 6-dichlorophenolindophenol and the other two categories by osazone formation. The possibility of interference by glutathione in the dye method was excluded by adding excess glutathione to a sample of blood and estimating its ascorbic acid content in parallel with an untreated sample of the same blood; no difference was found. However, the indophenol method is known to be susceptible to interference from reducing agents other than ascorbic acid (OWEN and IGGO, 1956) and an unknown change of this nature appears the most likely explanation of the observed arteriovenous difference.

DISCUSSION

The rabbit, to our knowledge, has not previously been used for arteriovenous studies of the brain. In the present work the feasibility of using this animal in a conscious state, is exemplified. It has afforded evidence of uptake of glucose and output of lactic acid by the brain which is comparable to findings in man. Results show, however, that the changes brought about in keto-acids at the brain (and to some extent the change in lactate) are not as regular as those in glucose. Possibly they are influenced by experimental circumstances not controlled in the present work, such as dietary or hormonal factors.

Turning to the other groups of substances studied, the present impression that many amino acids show no change in passing the brain must be considered as provisional only, since a change of less than 20 per cent in their values would not have been detected. Observations indicating an increase in the glutamine level of blood in

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passing the brain (ADAMS, GORDAN, HUTCHIN and BENTINECK, 1955) were reported while the present work was in progress. Although no definite change in the reduced and oxidized forms of ascorbic acid was found, the cerebral venous specimens apparently contained a substance interfering with the dichlorophenolindophenol method for estimating ascorbic acid; its nature remains to be investigated. With purine compounds, the probable loss of adenine from the cerebral venous blood of anaesthetized animals contrasts with the lack of change in normal animals, where arterial adenine levels were lower. The position is thus similar to that observed by BESSMAN, FAZEKAS and BESSMAN (1954) with respect to ammonia, which though normally unchanged at the brain is lowered in level in cerebral venous blood when, during hepatic coma, blood ammonia is high.

Preliminary reports (MARTIN, RODNIGHT, MCILWAIN and TRESIZE, 1957) of an oxidation of glutathione at the brain have proved incorrect (MCILWAIN, THOMSON and TRESIZE, 1957) but to afford a suggestion of the cerebral production of a substance affecting the glyoxalase assay first used in determining glutathione; investigation of the phenomena continues.

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SUMMARY

(1) Simultaneous samples of arterial and cerebral venous blood from anaesthetized and conscious rabbits were analysed for glucose, lactic acid, keto-acids, amino acids, indoles, purines, creatine and the reduced and oxidized forms of ascorbic acid.

(2) The mean arteriovenous difference in glucose in forty-seven experiments was 13·2 mg per cent. A small release of lactic acid by the brain was observed in four out of six animals but no change in blood keto-acids occurred on passing the brain.

(3) Using paper chromatographic methods no arteriovenous difference was observed in twenty amino acids and three indole derivatives (including 5-hydroxytryptamine). For amino acids changes of less than 20 per cent would not have been detected.

(4) The level of free adenine in arterial blood from anaesthetized rabbits was about 1·54 μg/ml, as compared with 0·6 μg/ml in conscious animals. Under anaesthesia there was a small arteriovenous difference in free adenine of 0·4 μg/ml, which was not found in conscious animals. No arteriovenous difference in nucleotide adenine or guanine was observed.

(5) The level of creatine in rabbit arterial blood was about 1·98 mg/ml; there was no arteriovenous difference at the brain.

(6) No arteriovenous difference at the brain in the total, oxidized or reduced forms of ascorbic acid was found, though a difference in associated material was suggested.

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THE INCORPORATION OF [^{35}S]LABELLED METHIONINE INTO THE PROTEINS OF THE RAT BRAIN

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GAITONDE and RICHTER (1953, 1955) reported that ^{35}S-labelled methionine is rapidly incorporated into the proteins of the rat brain *in vivo*. By determining the specific radioactivity in the acid-soluble fraction as well as in the isolated proteins of the brain they obtained evidence that, although the total amount of amino acid traversing the blood–brain barrier may be small, the rate of turnover of amino acids in the proteins of the brain is relatively high. The high metabolic activity of the brain proteins was confirmed in a further investigation in which the blood–brain barrier was by-passed by injecting the labelled amino acid directly into the cerebrospinal fluid (GAITONDE and RICHTER, 1956). It was found that, as BORSOOK *et al.* (1950) had previously shown for the liver and other organs, the half-life calculated for the mixed brain proteins depended on the time allowed for incorporation, being shorter in experiments of less than 60 minutes duration than in experiments of 3 and 5 hours. Calculations based on the uptake of [^{35}S]methionine *in vivo* during a period of 20–60 minutes indicated a mean half-life of the order of 14 days.

Since the proteins of the brain are not homogeneous, estimates of the mean rate of amino acid turnover in the mixed proteins of the whole brain are of limited significance. In the present investigation the proteins of the rat brain were fractionated in order to determine the nature of the proteins labelled most rapidly and to study the sequence of labelling in the various protein fractions at different times. Cell fractions containing nuclei, mitochondria, microsomes and soluble proteins were first separated by fractional centrifugation. The proteins in these cell fractions were then further separated into subfractions (A), (B) and (C) by successive extraction with sodium chloride and sodium hydroxide solutions. The results of this study have been described in a preliminary report (CLOUET and RICHTER, 1957): similar studies have recently been reported by KRAVCHINSKY and SILICH (1957) and by FURST, LAJTHA and WAELSCH (1958).

EXPERIMENTAL

[^{35}S]Methionine was obtained from the Radiochemical Centre, Amersham, with specific activity from 79 to 100 μc/mg. methionine. It was dissolved in physiological saline and, after neutralization to pH 7·0, it was diluted with 0·9% NaCl solution to a concentration giving 100 μc/0·1 ml.

Experimental animals. The rats were of the Wistar albino strain, bred in this laboratory from stock obtained originally from Messrs A. Tuck & Son, Rayleigh.

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The mean age of the 30 rats used in these experiments was 28 days, and the mean weight 37 g (ranging from 25 to 60 g). Each rat was injected intracisternally with DL-[^{35}S]methionine at a dose of 100 μC/100 g of body weight (contained in volumes of 0·025 to 0·060 ml of solution). The method of injection into the *cisterna magna* of the rat was described by GAITONDE and RICHTER (1956). The rats were observed until they were killed by decapitation and they did not show any adverse effects from the intracisternal injection.

The rats were divided into nine groups of 3 or 4 rats each. In series (I), 3 rats were killed 0·5 hr after the injection, 3 rats at 1 hr, 4 rats at 2 hr, 3 rats at 3 hr and 4 rats at 6 hr, and in series (II), groups of 3 rats each were killed at $\frac{1}{2}$, 1 and 3 hr, and 4 rats at 6 hr. The number of rats in each group was determined by the total body weight which was kept as close to 100 g as possible.

Preparation of the homogenate and fractions. After each rat was decapitated, the brain was removed, freed from blood by pressing with filter paper and weighed. The brains from all rats in a group were pooled and homogenized with a Potter–Elvehjem homogenizer in a volume of cold 0·25 M-sucrose sufficient to make a 10% suspension (10 g wet weight of brain/100 ml final volume). The homogenization, centrifugation, and all subsequent fractionation procedures were performed at a temperature of from 2 to 4°C.

An aliquot of 32 ml of the brain homogenate from each group in series (I) was fractionated by centrifuging (1) at 5000 **g** for 10 min, (2) at 12,000 **g** for 15 min and (3) at 23,000 **g** for 40 min. In series (II) the centrifugations were carried out (1) at 5,000 **g** for 10 min, (2) at 12,000 **g** for 15 min, and (3) at 45,000 **g** for 60 min. The cell fractions obtained by centrifugation cannot be regarded as homogeneous (see DALLAM, 1955), but they corresponded approximately to (1) nuclei and cell debris, (2) mitochondria, (3) microsomes, and (4) the non-sedimented or supernatant fraction. The composition of the fractions was checked by microscopic examination.

Each of the particulate fractions was further fractionated by extraction with aqueous solvents, followed by centrifugation at the speed and time originally used to prepare the fraction. In series (I) each particulate fraction was extracted successively with 0·1 M-NaCl, 1 M-NaCl, and 1 N-NaOH by homogenization with the solvent for 60 sec in an MSE homogenizer: this gave three soluble and one insoluble fractions. In series (II), the first extraction with 0·1 M-NaCl was omitted, and only two soluble and one insoluble fractions were obtained.

Preparation of fractions for analysis. Each of the soluble fractions was diluted with one-third volume of 40% (w/v) trichloroacetic acid (TCA) to give a 10% TCA solution. The insoluble fractions, as well as aliquots of the original particulate fractions, were suspended in 10% (w/v) TCA. An aliquot of the original homogenate was diluted with 20% TCA to produce a 10% solution. The TCA suspensions were centrifuged. The precipitates were washed twice with 10% TCA, then extracted for 10 min with 10 ml of chloroform : methanol (1 : 1), twice for 10 min with 10 ml of ether, and once for 10 min with 10 ml of acetone. The aqueous TCA supernatants and washings were pooled to give the non-protein fractions, and the chloroform : methanol, ether and acetone washings were pooled to give the lipid fraction. The washed precipitates were designated the protein fractions.

Chemical determinations. The protein fractions were dried at 110°C to constant weight. They were then separated into two portions (4–6 mg) for sulphur analysis

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and for hydrolysis, and into a further portion (1 mg) for a nitrogen determination by the micro-Kjeldahl method. The hydrolysis of protein was performed by heating the dry protein with 2 ml of 6N-HCl in a sealed tube in an oven at 110° for from 12 to 16 hr. The hydrolysate was neutralized and an aliquot was taken for the determination of amino-N (MOORE and STEIN, 1948) and nucleotide bases (LORING, FAIRLEY, BORTNER and SEAGRAN, 1952). The total nucleic acid was estimated by converting the sum of the purine and pyrimidines to the conventional tetranucleotide formula.

The lipid fractions were dried and weighed, then examined for the ratio of ^{35}S to total S. The TCA-soluble material was examined only in the supernatant fraction, in which the ratio ^{35}S/total S was obtained.

Determination of total sulphur and radioactivity. The determination of total sulphur, with the measurement of the radioactivity of the same sample, has been described in detail by GAITONDE and RICHTER (1955). The method consisted of an oxidation of the sample with perchloric–nitric acids and aqua regia–perchloric–nitric acids, followed by the precipitation of the sulphur as benzidine sulphate. The precipitated benzidine sulphate was collected in a uniform layer on filter paper and the ^{35}S was counted using an end-window Geiger counting tube. The benzidine sulphate was then suspended in distilled water and titrated against NaOH to obtain the amount of sulphate present.

RESULTS

The rate of transit of methionine across the blood–brain barrier. Shortly after intracisternal injection, the concentration of [^{35}S]methionine in the cerebrospinal fluid and in the brain is relatively high, but the concentration quickly falls as the labelled amino acid passes out through the blood–brain barrier into the blood stream and becomes distributed throughout the other tissues of the body. The rate of egress of methionine from the brain is indicated by the decrease in radioactivity of the non-protein supernatant fraction (Fig. 1), which includes the soluble metabolites in the amino acid 'pool' of the brain. KAMIN and HANDLER (1951) showed that methionine differs from most other amino acids in that it passes relatively easily through the blood–brain barrier into the brain: but little information is available in the literature about the rate at which amino acids can pass through the blood–brain barrier in the reverse direction out of the brain. In the present experiments it was found that for a mean injected dose of 370 μg of [^{35}S]methionine only 2·1 per cent of the radioactivity remained in the non-protein supernatant fraction at 30 min after the injection. If it is taken that the amount of ^{35}S incorporated in the proteins was 0·23 per cent of the total, and the mean amount of methionine metabolized was 2 per cent in that time (GAITONDE and RICHTER, 1956), it may be concluded that the amount of methionine passing out of the brain was about 354 μg in 30 min. This would correspond to a rate of egress of 4·8 μmoles/hr from the rat brain under these conditions.

At 60 min after intracisternal injection only 1·3 per cent of the radioactivity in the injected dose of [^{35}S]methionine remained in the non-protein supernatant fraction of the brain. The present data are compared in Table 1 with values previously reported for the rate of egress of labelled L-methionine and γ-aminobutyric acid from the brain after intracisternal injection. Although GAITONDE and RICHTER (1956)

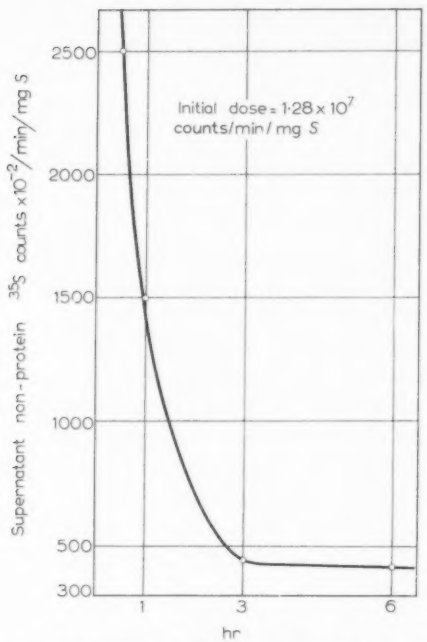

FIG. 1.—The rate of egress of [^{35}S]methionine from the brain of the rat.

injected almost five times the amount of methionine used in the present experiments, the proportions of the injected radioactivity remaining in the soluble fraction at 30 and 60 min were approximately the same. This suggests that methionine in the brain and cerebrospinal fluid quickly comes into equilibrium via the blood with that in the other tissues: there is no evidence that a blood–brain barrier plays any part in

TABLE 1.—THE RATE OF EGRESS OF AMINO ACIDS FROM THE BRAIN. SHOWING THE PROPORTION OF THE TOTAL RADIOACTIVITY INJECTED THAT REMAINED IN THE SOLUBLE FRACTION OF THE BRAIN AT DIFFERENT TIMES AFTER INTRACISTERNAL INJECTION

Time after injection (min)	Proportion of total radioactivity remaining in the brain (%)		
	L-Methionine* (11·4 μmoles)	DL-Methionine† (2·5 μmoles)	γ-Aminobutyric acid‡ (2·4 μmoles)
5	—	—	20·9
15	8·0	—	—
30	1·8	2·1	5·9
60	1·0	1·3	1·1

*L[^{35}S]methionine in rats of 40 g mean weight (GAITONDE and RICHTER, 1956).
†Present data.
‡Percentage remaining in the mouse brain as [^{14}C]γ aminobutyric acid (ROBERTS, ROTHSTEIN and BAXTER, 1958).

preventing the free egress of methionine from the brain. The values for the proportion of free γ-aminobutyric acid remaining in the brain show a similar rapid transit of the amino acid across the blood–brain barrier and out of the brain. From these experiments, and the observations of KAMIN and HANDLER (1951), it may be concluded that the blood–brain barrier offers very little hindrance to the passage of methionine in either direction into or out of the brain. The intracisternal route of injection was of value in the present investigation in giving a high initial concentration of the labelled compound in the cerebrospinal fluid and the blood, which are the two physiological routes of entry of metabolites into the brain. A high overall incorporation of the label into the brain fractions was thereby achieved.

Chemical characteristics of the fractions. In Table 2 are shown the results of the chemical analyses of the fractions. The values for the homogenate and for all the nuclear and mitochondrial fractions are mean values based on data obtained from nine groups of rats in series (I) and (II). The values for the microsomal and supernatant fractions are taken from five groups of rats in series (II) only: a better separation of these two fractions was obtained by the use of an higher centrifugal force to prepare the microsomal fraction in series (II).

Subfraction (A) is saline-soluble (including material soluble in 0·1 N- and N-NaCl in series (I). Subfraction (B) is alkali-soluble and subfraction (C) is the insoluble residue. The values for the particulate fractions and for the original homogenate were obtained from separate analyses and they are not the sums of the component subfractions.

Since complete tissue proteins are characterized more satisfactorily by their content of non-protein components than by their amino acid composition, the proportions of lipid and nucleic acid to protein in each fraction were determined. The lipoproteins in the particulate fractions were not generally extracted by saline or alkali to the same extent as the other proteins, so that material with a higher lipid/protein ratio was left in the residual fractions. On the basis of the purine and pyrimidine analyses, the nucleoprotein content was highest in the saline-soluble portions of the nuclear and mitochondrial fractions, and in the alkali-soluble subfraction derived from the microsomes.

It is difficult to obtain a quantitative separation of lipids and proteins by simple extraction procedures such as those described, since even after treatment with trichloroacetic acid and prolonged extraction with organic solvents, the removal of lipid material is still incomplete. At the same time, it is known that an appreciable amount of proteolipid protein passes into the lipid solvents under these conditions. In order to exclude possible errors due to these causes, the sulphur content and specific activity of the sulphur in the lipid extracts of each fraction were determined. In view of the low sulphur content of the lipid fractions, and the difficulty of removing all traces of adsorbed amino acids, the values in Table 3 are given with considerable reserve, but they serve to show that the specific activity of the lipid fraction is relatively low and that any errors due to the incomplete removal of lipid from the proteins are likely to be small. Since lipid S may be expected to represent mainly proteolipid S (GAITONDE and RICHTER, 1957) the data indicate that the rate of incorporation of [^{35}S]methionine in the proteolipids must be relatively slow. This is particularly evident in the microsomal lipid fraction, which gives the lowest rate of uptake of [^{35}S]methionine.

TABLE 2.—CHEMICAL CHARACTERISTICS OF CELL FRACTIONS FROM RAT BRAIN
(mg/g of fresh tissue ± S.D.)

Cell fraction	Sub-fraction	Lipid extract (dry wt.)	Ratio lipid/protein (%)	Protein residue				Nucleic acid	Ratio nucleic acid/protein (%)
				Dry wt.	Nitrogen	Amino-nitrogen	Sulphur		
Whole homogenate		86·3 ± 7·3	97	98·9 ± 13·7	13·1 ± 1·9	7·1 ± 1·7	1·59 ± 0·40	1·68 ± 0·05	1·9
Nuclei		39·6 ± 10·8	78	50·2 ± 15·6	7·4 ± 1·2	4·6 ± 1·2	0·89 ± 0·31	0·87 ± 0·06	1·7
Nuclei	A	13·7 ± 6·7	107	15·1 ± 4·2	1·9 ± 0·4	1·2 ± 0·3	0·36 ± 0·24	0·33 ± 0·03	2·6
Nuclei	B	15·1 ± 2·6	64	25·7 ± 10·4	3·6 ± 0·25	2·8 ± 0·7	0·37 ± 0·14	0·51 ± 0·09	2·1
Nuclei	C	7·7 ± 2·9	202	5·9 ± 1·6	0·6 ± 0·25	0·3 ± 0·0	0·16 ± 0·11	0·06 ± 0·01	1·6
Mitochondria		20·6 ± 7·1	103	20·5 ± 4·4	2·9 ± 1·0	1·07 ± 0·31	0·39 ± 0·13	0·38 ± 0·05	1·9
Mitochondria	A	8·5 ± 2·4	171	7·3 ± 3·1	0·7 ± 0·25	0·40 ± 0·13	0·12 ± 0·06	0·13 ± 0·00	2·6
Mitochondria	B	7·8 ± 4·6	98	9·0 ± 3·7	1·2 ± 0·5	0·36 ± 0·22	0·16 ± 0·03	0·17 ± 0·09	2·1
Mitochondria	C	5·5 ± 2·9	145	3·6 ± 1·7	0·6 ± 0·3	0·45 ± 0·09	0·05 ± 0·02	0·05 ± 0·02	1·3
Microsomes		12·5 ± 6·8	287	6·2 ± 2·2	0·64 ± 0·27	0·24 ± 0·02	0·11 ± 0·04	0·09 ± 0·02	2·1
Microsomes	A	3·5 ± 1·5	206	2·1 ± 1·7	0·25 ± 0·02	0·05 ± 0·02	0·05 ± 0·02	0·02 ± 0·00	1·2
Microsomes	B	2·0 ± 0·6	112	2·2 ± 1·3	0·25 ± 0·02	0·04 ± 0·02	0·03 ± 0·01	0·06 ± 0·00	3·6
Microsomes	C	6·5 ± 2·0	281	2·3 ± 0·9	0·34 ± 0·18	0·14 ± 0·04	0·04 ± 0·01	0·01 ± 0·00	0·4
Supernate		13·1 ± 4·8	94	19·9 ± 5·4	2·0 ± 0·4	1·05 ± 0·37	0·21 ± 0·08	0·34 ± 0·06	2·5

The data are mean values from five to nine experiments ± standard deviations. The ratios are based on protein figures obtained by multiplying the mean nitrogen values by 6·8. This factor has been shown to be more accurate than the conventional factor 6·25 for converting nitrogen values to protein in the brain of the young rat (CLOUET and GAITONDE, 1956).

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TABLE 3.—SPECIFIC RADIOACTIVITY OF SULPHUR IN THE LIPID FRACTIONS FROM RAT BRAIN

Cell fraction	Subfraction	Lipid S (mg/100 g fresh brain)	Specific activity of lipid S (counts/min × 10^{-2}/mg S)		
			1 hr	3 hr	6 hr
Homogenate		10·6	80	97	107
Nuclei		4·8	72	84	115
Nuclei	A	1·2	93	—	103
Nuclei	B	0·9	71	101	125
Nuclei	C	2·4	59	109	140
Mitochondria		2·7	14	11	103
Mitochondria	A	1·1	8	19	15
Mitochondria	B	0·5	10	18	121
Mitochondria	C	1·3	40	9	81
Microsomes		2·0	18	21	28
Microsomes	A	0·6	4	3	13
Microsomes	B	0·5	2	15	19
Microsomes	C	0·4	74	80	72
Supernate		1·7	104	198	119

Turnover of [35S]*methionine in the protein fractions*

The radioactivity of the protein fractions was expressed both as the *specific activity* (counts/min/per mg total S) and as the *relative specific activity* (here defined as *specific activity of the protein/specific activity of the non-protein supernate*). This latter term is analogous to the ratio *specific activity of the protein/specific activity of the trichloroacetic acid-soluble fraction*, used by GAITONDE and RICHTER (1955) to express the rate of incorporation of [35S]methionine into brain fractions.

The specific activities of the homogenate and the nuclear and mitochondrial fractions of series (II) agreed closely with those obtained in series (I). Owing to the higher speed of the final centrifugation in series (II), the supernatant fraction in series (II) had lower, and the microsomal fraction higher, specific activities than those in series (I). The microsomal fraction in series (II) was prepared under conditions more closely approximating to those generally used to obtain microsomes from other tissues such as liver and pancreas, and the data from series (II) only are given in Tables 2, 3 and 4.

It may be seen that at 0·5, 1 and 3 hr after the injection of [35S]methionine, the incorporation of the labelled amino acid was highest in the microsomal cell fraction, and at 6 hr after the injection it was highest in the supernatant fraction. The labelling of all of the microsomal subfractions (A), (B) and (C) was higher than in the original homogenate for the first 3 hr, with the highest activity in the alkali-soluble subfraction (B) (Table 4). At 6 hr the labelling was still high in this same microsomal subfraction: it was also relatively high in the saline-soluble subfractions (A) of the nuclear and mitochondrial proteins and in the supernatant protein.

It is significant that during the period from 3 to 6 hr after injection of [35S]methionine, the specific activity of the proteins in the nuclear, mitochondrial and supernatant fractions continued to rise, although the specific activity in the

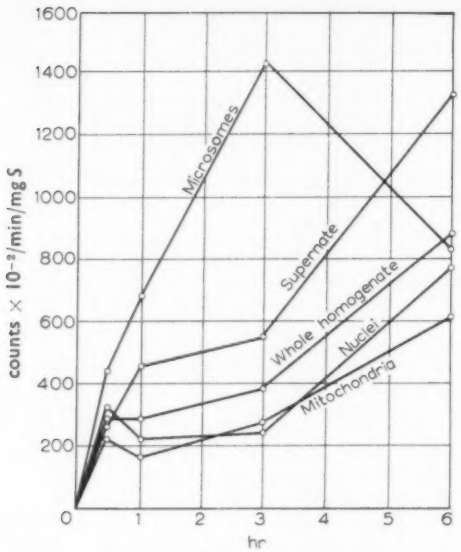

FIG. 2.—The incorporation of [^{35}S]methionine into sub-cellular fractions of the rat brain.

TABLE 4.—SPECIFIC RADIOACTIVITY OF THE PROTEIN FRACTIONS FROM RAT BRAIN AT DIFFERENT TIMES AFTER INJECTION OF (^{35}S)METHIONINE

Cell fraction	Sub-fraction	Specific activity (counts/min × 10^{-2}mg S)				Relative specific activity (counts/min per mg S in protein fraction/ counts/min per mg S in non-protein supernate)			
		0·5 hr	1 hr	3 hr	6 hr	0·5 hr	1 hr	3 hr	6 hr
Homogenate		248	279	376	885	0·009	0·276	0·840	2·105
Nuclei		218	215	241	779	0·087	0·213	0·539	1·850
Nuclei	A	276	262	224	894	0·110	0·260	0·500	2·122
Nuclei	B	212	221	291	812	0·085	0·220	0·650	1·932
Nuclei	C	98	72	203	206	0·039	0·071	0·453	0·490
Mitochondria		204	169	276	612	0·082	0·208	0·615	1·455
Mitochondria	A	268	164	319	1108	0·107	0·163	0·711	2·630
Mitochondria	B	194	167	185	516	0·077	0·166	0·412	1·227
Mitochondria	C	177	164	216	483	0·071	0·163	0·481	1·150
Microsomes		439	686	1432	836	0·176	0·678	3·190	1·985
Microsomes	A	350	688	810	742	0·138	0·680	1·180	1·765
Microsomes	B	445	1164	1675	1408	0·178	1·435	3·740	3·350
Microsomes	C	362	506	889	396	0·145	0·623	1·982	0·940
Supernate		273	451	544	1335	0·109	0·446	1·214	3·170
Supernate non-protein		2500	1012	448	421	—	—	—	—

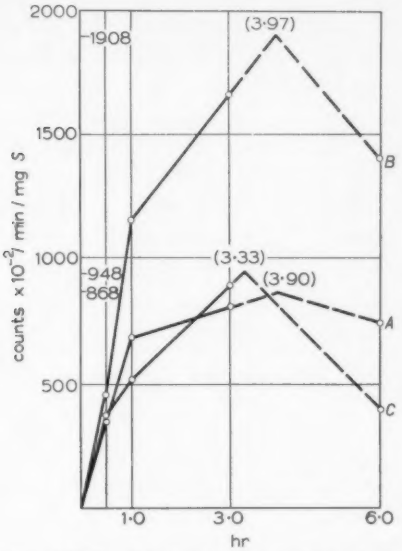

FIG. 3.—Incorporation of [35S]methionine into the proteins of microsomal subfractions of the rat brain. (A) Protein extracted by M-NaCl from the microsomes. (B) Protein obtained by further extraction with N-NaOH from the residue of (A). (C) Residual protein after extraction with M-NaCl and N-NaOH.

soluble non-protein fraction of the supernate had fallen to a relatively low value. The increase in specific activity in the proteins of these three fractions coincided with a sharp decrease of specific activity in the microsomal fraction (Fig. 2). The relations, which are shown more clearly in the relative specific activity values (Table 4) suggest a primary labelling of the microsomal proteins and a subsequent transfer of labelled amino acid to the proteins in the other cell fractions.

An estimate of the half-life of the proteins in the microsomal subfractions (A), (B) and (C) can be made from the specific activity curves by assuming a symmetrical peak and interpolating to obtain the time of maximal specific activity (t_{max}) as indicated in Fig. 3. The half-life ($t_{1/2}$) is then obtained by applying the formula:

$$t_{1/2} = \ln 2 \frac{I_{1/2max}}{I_{max}} t_{max}$$

Calculations made in this way indicated relatively short half-lives of the order of 58–128 min for the proteins in the microsomal subfractions (Table 5). These figures may be compared with estimates calculated in another way from the time required for complete replacement of the methionine in the fraction, if the rate for the first 30 min continued until all the protein-bound methionine was replaced. While neither of these methods can be regarded as giving more than an approximate estimate, they agree in indicating that proteins in the microsomal subfractions have a mean half-life that is quite short and of the order of 50 to 100 min.

DISCUSSION

Measurements of the rate of incorporation of [35S]methionine into different protein fractions of the rat brain *in vivo* have shown that for a short period of incorporation of 30 minutes, the uptake of labelled amino acid is highest in the proteins

of the microsomal fraction (CLOUET and RICHTER, 1957). At 30 minutes the labelling of the nuclear, mitochondrial and soluble proteins of the supernatant fraction was still relatively small. These observations on the brain agree with previous reports for liver and other actively metabolizing tissues in which the microsomal proteins were shown to be the most rapidly labelled (BORSOOK *et al.*, 1950; DALY, ALLFREY and MIRSKY, 1955; LITTLEFIELD *et al.*, 1955). In a similar study KRAVCHINSKY and SILICH (1957) have also found an active labelling of the microsomal fraction of the rat brain, and FURST *et al.* (1958) have recently reported an active labelling of the microsomal proteins of the monkey brain after intravenous injection of ^{14}C-lysine.

TABLE 5.—THE CALCULATED HALF-LIFE OF THE SUBFRACTIONS OF THE MICROSOMAL PROTEINS

	Subfraction (A)	Subfraction (B)	Subfraction (C)
(a) Graphical method			
t_{max} (hr)	3·90	3·97	3·33
half-life (min)	128	94	58
(b) Replacement method			
complete (hr)	6·3	2·87	4·13
half-life (min)	112	50	74

In (a) the values are calculated from the interpolated curves shown in Fig. 3.
In (b) the values are calculated from the time required for complete replacement of the methionine in the protein of the fraction, if the rate for the first 30 min continued until the total protein-bound methionine was replaced.

In an attempt to characterize more clearly the proteins with the highest turnover rate, the proteins of the nuclei, mitochondria and microsomes of the rat brain have been further fractionated by successive extraction with saline and with sodium hydroxide solutions. The highest rate of labelling was found in an alkali-soluble lipoprotein obtained from the microsomes in subfraction (B). It contains nearly equal parts of protein and lipid material (112 g lipid/100 g protein, or 53 % (w/w) lipid in the lipoprotein). This protein fraction also has a relatively high nucleic acid content corresponding to 3·6 g nucleic acid per 100 g protein (or 1·8 per cent of the lipoprotein). It thus has the composition of a liponucleoprotein, and it may be expected to correspond to the lipid-nucleoprotein aggregate forming the Nissl granules in stained sections of brain tissue.

Estimates of the rate of turnover of [^{35}S]methionine in the microsomal liponucleoprotein (subfraction (B)) from the specific radioactivity–time curves gave a half-life of the order of 94 minutes. This is of the same order as an estimate of 50 minutes for the half-life of the protein in this fraction calculated in another way from the rate of incorporation during the first 30 minutes. Short half-lives of a similar order were found also for the proteins in the saline-soluble subfraction (A) of the microsomes (112 and 128 minutes) and in the residual insoluble subfraction (C) (58 and 74 minutes). Subfraction (C) contains a relatively high proportion of lipids. The protein subfractions (B) and (C) of the microsomes may be related to the desoxycholate-soluble and insoluble fractions of rat liver microsomes, which show similar time relationships, although the labelling is more rapid in liver microsomes (LITTLEFIELD *et al.*, 1955).

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The present study gives evidence of a steady increase in the labelling of the more slowly metabolizing proteins in the nuclear, mitochondrial and supernatant fractions during the period from 3 to 6 hours after injection in spite of a greatly reduced availability of the labelled amino acid in the amino acid 'pool' of the tissue during that period. This would be consistent with a primary uptake of the labelled amino acid into the microsomal liponucleoprotein of fraction (B) and a subsequent transfer to the other protein fractions of the cell.

SUMMARY

(1) Brain homogenates from the rat were separated by centrifugation into particulate fractions. The proteins in these cell fractions were then further fractionated by successive extraction with M-NaCl and M-NaOH. The chemical composition of the proteins in the original particulate and non-sedimented fractions and in the subfractions was determined, as were the lipid/protein and nucleic acid/protein ratios.

(2) The rate of incorporation of [^{35}S]methionine into the protein fractions of the brain of young rats was studied at times from ½ hour to 6 hours after the intracisternal injection of the labelled amino acid. The highest specific activity at from ½ to 3 hours after the injection was found in the protein of a microsomal fraction extractable with alkali. It had the properties of a liponucleoprotein. The calculated half-life of this protein was of the order of from 50 to 94 minutes.

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THE RESULTS OF GLYCOLIPID ANALYSIS IN CERTAIN TYPES OF LIPIDOSIS AND LEUCODYSTROPHY

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THE increasing interest recently shown in the chemical aspects of metabolic disorders of the nervous system (CUMINGS, 1957) has prompted the present investigation of the glycolipid fractions in cases of cerebral lipidosis and leucodystrophy. The histological features of these disorders are now well known and the possibility of further advances in knowledge doubtless lies in the field of chemical pathology. Confusion has arisen from the use of a great variety of methods, some of which are known to be inaccurate, thus rendering comparison of results obtained in different laboratories difficult.

The estimation of galactose, hexosamine and neuraminic acid can now be considered reasonably accurate, particularly since the introduction of SVENNERHOLM's (1957) method for estimating neuraminic acid. The calculations of ganglioside and cerebroside based on these results, are, however, open to serious criticism. In spite of these obvious shortcomings we believe with EDGAR (1958) that biochemistry can make important contributions to routine neuropathology.

EXPERIMENTAL

Materials

The material consisted of eight cases of cerebral lipidosis, five cases of leucodystrophy and four normal brains.

(1) *The lipidoses*: Two cases of infantile amaurotic idiocy of the Tay–Sachs' type. One, in which only a very limited amount of material was available had been preserved in formalin for several months (a), the other (b) aged 17 months only for 18 hr.

Three cases of amaurotic idiocy of the Batten type: two juvenile cases, one of which (a) aged 24 years had been preserved in formalin for 23 days, the other (b) aged 15 years was unfixed; also one example of the precocious ('late infantile') (c) variety aged 5 years and preserved in formalin for 108 days (reported by TINGEY, NORMAN, URICH and BEASLEY, 1958).

Two cases of gargoylism, one (a) aged 5 years preserved in formalin for 4 years (reported by BISHTON, NORMAN and TINGEY, 1956) the other (b) aged 19 years, fixed for 1 year.

One case of Niemann–Pick's disease aged 18 months, in formalin for 1 year.

(2) *The leucodystrophies:* Two cases (siblings) of the Krabbe or globoid cell type; one (a) aged 18 months in formalin for 6 weeks, the other (b) aged 8 months, unfixed.

Two cases (siblings) of metachromatic leucodystrophy; one (a) aged 3 years in formalin for 9 months, the other (b) aged 3 years, unfixed.

One case of Pelizaeus–Merzbacher's disease, aged 2 years, in formalin for 5 months.

The diagnosis in all these cases has been histologically verified in this laboratory.

(3) *Normal controls*. Brains from two adults aged 58 years and 65 years, from a newly born infant and from a child of 2½ years. The first three were unfixed, the last had been in formalin for 4 years.

Methods

(1) *Extraction*

Dried tissue was weighed and ground to a fine powder with a small quantity of acid-washed sand, refluxed for 3 hr with MeOH : $CHCl_3$ (2 : 1 v/v), filtered and again extracted for 1 hr with $CHCl_3$

alone. The combined extract was taken down to a low volume, made up to 50 ml, and aliquots taken for analysis. A separate extract was prepared for neuraminic acid estimation after the method of SVENNERHOLM (1957).

(2) *Estimations*

Total lipid galactose. Estimated after BRAND and SPERRY (1941).

Neuraminic acid. Estimated after SVENNERHOLM's (1957) modification of KLENK and LANGERBEINS' (1941) method. A sample of *N*-acetylsialic acid provided by SVENNERHOLM with a molar absorbancy index 102 per cent that of neuraminic acid was used as a standard and the appropriate correction applied.

Hexosamine. Estimated after BLIX (1948). The following fractions were examined:

Total hexosamine: the original dried tissue was hydrolysed for 8 hr with 4N–HCl.

Lipid hexosamine: the lipid extract was hydrolysed for 2 hr with 3N-HCl. Blank values were obtained by similar treatment, except that 1 ml conc. HCl + 1 ml H_2O were substituted for the 2 ml *p*-dimethylaminobenzaldehyde solution in the final stage.

Water-soluble hexosamine (BRANTE, 1957). The protein residue was extracted with water on a boiling-water bath and water-soluble mucopolysaccharide precipitated with excess of ethyl alcohol slightly acidified with acetic acid. After washing with ether, the precipitate was hydrolysed for 2 hr with 3N-HCl and the hexosamine assessed as above.

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(3) *Calculations*

Ganglioside was calculated from the neuraminic acid after SVENNERHOLM (1957) by multiplying the neuraminic acid value by a factor of 4 (ganglioside N.A.). It was also calculated from lipid hexosamine after EDGAR (1955) by multiplying the lipid value by a factor of 8·33 (ganglioside Hex.).

Ganglioside galactose was calculated from the respective ganglioside values by dividing by 2·48.

Cerebroside was calculated in three ways:

(a) Neuraminic acid ganglioside galactose was subtracted from total lipid galactose and the remainder multiplied by a factor of 4·55 (cerebroside N.A.).

(b) Hexosamine ganglioside galactose was subtracted from total lipid galactose and the remainder multiplied by a factor of 4·55 (cerebroside Hex.).

(c) The total lipid galactose was multiplied by a factor of 4·55 (cerebroside total galactose). This method is known not to represent true cerebroside, but is introduced for comparison as it is still very widely used.

RESULTS

The estimated values of lipid galactose, neuraminic acid, the hexosamine fractions and the water content of the tissue are shown in Table 1. The value of residue hexosamine was obtained by subtraction of the lipid hexosamine from the total.

The values of ganglioside and cerebroside calculated by various methods are shown in Table 2.

DISCUSSION

Satisfactory analysis of the glycolipid fractions largely depends on the existence of a reliable method for the estimation of ganglioside. The classical method of KLENK and LANGERBEINS (1941) which has yielded valuable results, particularly in the investigation of Tay–Sachs' disease, has certain well-known limitations. The presence of myelin lipids interferes with the reaction which therefore yields negative results in the normal white matter. Furthermore, according to SVENNERHOLM (1957), a considerable loss of ganglioside, up to 60 per cent, may occur in the ether fraction during the process of elimination of myelin lipids. The results obtained by KLENK's method are therefore too low. In our experience the loss of ganglioside in this method is very variable, even in normal controls. In addition, it seems that the loss may be more severe in normal cases than in some pathological material, a fact which may be responsible for some misinterpretation of the figures obtained by this method.

TABLE 1

% dry tissue	Cortex							White matter						
	Total lipid galactose	N.A.	Lipid hex.	Residue hex. (by difference)	H_2O-sol. hex.	Total hex.	H_2O	Total lipid galactose	N.A.	Lipid hex.	Residue hex. (by difference)	H_2O-sol. hex.	Total hex.	H_2O
Controls:														
Adult, fresh, 58 yr	1·26	0·485	0·182	0·513	0·043	0·695	86·7	2·90	0·130	0·052	0·288	0·009	0·340	71·1
Adult, fresh, 65 yr	0·970	0·451	0·134	0·480	0·050	0·614	83·2	2·77	0·106	0·041	0·172	0·015	0·213	70·7
2½ yr, formalin	1·12	0·490	0·137	0·156	—	0·293	88·0	2·02	0·096	0·057	0·228	—	0·285	76·4
Newborn, fresh	0·770	0·306	0·124	0·656	0·075	0·780	87·7	0·835	0·431	0·192	0·448	0·075	0·640	87·2
Amaurotic family idiocy:														
Tay–Sachs' (a)	3·80	—	1·12	—	—	—	—	3·34	0·872	0·910	0·550	—	1·46	—
Tay–Sachs' (b)	2·50	1·47	0·672	0·988	0·253	1·66	83·5	3·26	1·21	0·870	0·520	0·125	1·39	85·0
Batten (a)	1·30	0·392	0·168	0·522	—	0·690	86·3	1·76	0·054	0·031	0·254	—	0·285	78·3
Batten (b)	0·790	0·311	0·131	0·511	0·048	0·642	86·2	1·60	0·132	0·049	0·301	0·006	0·350	74·0
Batten (c)	0·374	—	0·090	0·560	—	0·650	81·4	0·680	0·105	0·051	0·344	—	0·395	89·0
Niemann–Pick	1·78	0·482	0·180	0·370	—	0·550	81·4	0·967	0·296	0·148	0·472	—	0·620	81·7
Gargoyle (a)	1·45	0·483	0·197	0·428	—	0·625	85·7	2·08	0·080	0·061	0·394	—	0·455	—
Gargoyle (b)	1·50	0·549	0·192	0·548	—	0·740	86·6	2·75	0·093	0·043	0·328	—	0·372	78·0
Leucodystrophy:														
Krabbe (a)	1·01	0·384	0·123	0·449	—	0·572	87·8	0·990	0·106	0·049	0·331	—	0·380	82·4
Krabbe (b)	0·770	0·426	0·197	0·603	0·085	0·800	87·8	1·12	0·286	0·145	0·545	0·028	0·690	84·7
Metachromatic (a)	1·01	0·524	0·189	0·341	—	0·530	84·8	2·59	0·106	0·092	0·443	—	0·535	83·3
Metachromatic (b)	1·56	0·502	0·176	0·544	0·043	0·720	92·1	2·44	0·176	0·132	0·443	0·128	0·575	94·0
Pelizaeus–Merzbacher	0·990	0·412	0·138	0·632	—	0·770	86·3	0·792	0·368	0·115	0·655	—	0·770	88·8

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TABLE 2

% dry tissue	Cortex					White matter				
	Ganglioside N.A.	Ganglioside hex.	Cerebroside N.A.	Cerebroside hex.	Cerebroside (tot. gal.)	Ganglioside N.A.	Ganglioside hex.	Cerebroside N.A.	Cerebroside hex.	Cerebroside (tot. gal.)
Controls:										
Adult, fresh, 58 yr	1·84	1·52	2·22	2·36	5·7	0·52	0·43	12·25	12·40	13·2
Adult, fresh, 65 yr	1·80	1·11	1·11	2·33	4·4	0·42	0·34	11·80	11·90	12·6
2½ yr formalin	1·96	1·14	1·50	3·00	5·1	0·38	0·47	8·50	8·33	9·2
Newborn, fresh	1·22	1·03	1·26	1·62	3·5	1·72	1·59	0·65	0·89	3·8
Amaurotic family idiocy:										
Tay–Sachs' (a)	—	9·35	—	0·14	17·3	3·49	7·57	8·77	1·27	15·2
Tay–Sachs' (b)	5·88	5·58	0·55	1·14	11·4	4·84	7·25	5·95	1·55	14·8
Batten (a)	1·57	1·40	3·04	3·34	5·9	0·22	0·26	7·60	7·50	8·0
Batten (b)	1·24	1·09	1·34	1·59	3·6	0·53	0·41	6·30	6·50	7·3
Batten (c)	—	0·75	—	0·32	1·7	0·42	0·42	2·32	2·32	3·1
Niemann–Pick	1·93	1·50	4·55	5·35	8·1	1·18	1·23	2·24	2·14	4·4
Gargoyle (a)	1·99	1·64	3·06	3·60	6·6	0·32	0·51	8·85	8·50	9·5
Gargoyle (b)	2·20	1·59	2·80	3·92	6·8	0·37	0·36	11·80	11·85	12·5
Leucodystrophy:										
Krabbe (a)	1·54	1·03	1·77	2·70	4·6	0·42	0·41	3·74	3·75	4·5
Krabbe (b)	1·70	1·64	0·39	0·50	3·5	1·14	1·21	3·00	2·88	5·1
Metachromatic (a)	2·10	1·57	0·75	1·72	4·6	0·42	0·76	11·00	10·40	11·8
Metachromatic (b)	2·01	1·46	3·42	4·42	7·1	0·70	1·10	9·80	9·10	11·1
Pelizaeus–Merzbacher	1·65	1·15	1·48	2·39	4·5	1·47	0·96	0·91	1·84	3·6

The introduction of SVENNERHOLM's (1957) modification which reduces the loss of ganglioside to a negligible minimum, enables us now to estimate neuraminic acid in the cerebral cortex with a considerable degree of accuracy. Positive readings are also obtained in the white matter, but here it has not been possible to eliminate interfering substances altogether. The results are therefore less accurate than in the cortex.

To obviate the difficulties arising out of KLENK and LANGERBEINS' method, SMITS and EDGAR (1958) suggested an alternative method of calculating ganglioside from the lipid hexosamine content of the material. The validity of this method is based on the observation that there is a rough parallelism between the levels of ganglioside and hexosamine both in normal and pathological material.

In the present material we have used both EDGAR's and SVENNERHOLM's methods of calculating ganglioside. We can confirm EDGAR's observation that in the cerebral cortex there is a rough parallelism between the figures obtained by both methods. In accordance with SVENNERHOLM's observations, the figures based on neuraminic acid estimations are slightly higher than those obtained for hexosamine estimations. The reason for this discrepancy is not entirely clear. It may be due to an inherent error of the method, or to the presence of a substance with a slightly different structural formula than that provisionally suggested for ganglioside by KLENK (quoted by EDGAR, 1956). This substance may contain neuraminic acid, but not hexosamine, or may contain both components in a different proportion.

It is also possible that the difficulty lies in the choice of the conversion factor. Opinions on the structure of gangliosides and their hexosamine content vary considerably. BRANTE (1957) gives the hexosamine content as 7–8 per cent, while BOGOCH (1958) in the latest work on the structure of ganglioside states that the hexosamine content is 10 per cent. It is therefore probable that the conversion factor for hexosamine ganglioside on which our calculations are based may be too low.

The position as regards the white matter is somewhat different. According to SVENNERHOLM the results obtained by both methods are approximately identical in normal brains in which satisfactory estimations have been made. In the pathological material here presented there is a considerable excess of 'hexosamine ganglioside' over 'neuraminic acid ganglioside' in several instances. It remains an open question whether this discrepancy should be ascribed to the inadequacy of the method for estimating neuraminic acid in the white matter; to the effect of formalin (KLENK *et al.*, 1957) or to the presence of a substance containing hexosamine, but not neuraminic acid. Such a substance might be either a glycolipid similar to that identified by KLENK and LAUENSTEIN (1951, 1952) in human erythrocytes or a non-lipid substance which, if present in considerable excess, may be 'co-extracted' in lipid solvents (SMITS and EDGAR, 1958).

The estimation of cerebroside is closely linked with the estimation of ganglioside. This problem has been recently investigated by SVENNERHOLM (1956) and it is therefore only necessary to discuss the application of his conclusions to pathological material. SVENNERHOLM points out that as long as the composition of gangliosides and allied substances is not accurately known, all calculation methods based on deduction of ganglioside hexose from the total estimated hexose depend on certain arbitrary assumptions. He therefore favours a two-phase separation process which avoids this difficulty. His method, however, is not applicable to material which contains an excess of ganglioside as in these cases a considerable proportion of ganglioside may

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remain in the lipid phase. This precludes the use of the method for pathological material. The same reservations apply to the essentially similar method of FOLCH *et al.* (1951). RADIN, LAVIN and BROWN (1955) offer an alternative method of estimating the neutral cerebrosides after removal of acid glycolipids by absorption on alkaline resins. This method promises a higher degree of accuracy, but its application to pathological material requires further investigation. It is therefore more expedient at present to use calculation methods provided it is understood that they can yield at best only approximations. Furthermore, it may be pointed out that the figure obtained after deduction of ganglioside does not represent pure cerebroside, but includes sulphatides and such non-lipid contaminants as are extracted by lipid solvents.

The point arises here whether the ganglioside hexose to be deducted should be calculated from the values obtained by estimation of neuraminic acid or those obtained by estimation of hexosamine. For the cerebral cortex calculations based on neuraminic acid are to be preferred because of the high degree of accuracy of SVENNERHOLM's method for the cortex and because cerebroside figures obtained in this way correspond closely with those obtained by the two-phase separation process. In the white matter, on the other hand, calculation from hexosamine values appears to be more satisfactory. Here the neuraminic acid estimations may not be entirely reliable. Whatever the nature of the hexosamine containing substance may be, whether glycolipid or polysaccharide, it probably contains a certain amount of hexose which should be deducted from the estimated hexose. The application of the same conversion factor to these hypothetical substances as to ganglioside is of course purely arbitrary. Nevertheless, the method yields results comparable with those of KLENK (1953) in cases of Tay–Sachs' disease in which the alternative method of calculation based on neuraminic acid would produce unduly high cerebroside figures.

If we now turn to the interpretation of the figures obtained by these methods, the following conclusions emerge. A considerable increase in neuraminic acid both in the cortex and in the white matter has been found only in the Tay–Sachs' form of amaurotic family idiocy. In addition, a large amount of hexosamine in all fractions was found in these two cases. This finding suggests that in addition to a disturbance in glycolipid metabolism an error of polysaccharide metabolism may also be present in this disease.

A smaller, but definite increase in the amount of neuraminic acid was found in the case of Niemann–Pick's disease, particularly in the white matter. Variable degrees of ganglioside storage in addition to the predominant sphingomyelin storage have been found in this condition by various writers and it is worth noting that the first discovery of a cerebral glycolipid other than cerebroside ('substance X') was made in a case of Niemann–Pick's disease (BAUMANN, KLENK and SCHEIDEGGER, 1936).

No increase in neuraminic acid was found in cases of the Batten type of amaurotic family idiocy, either in the classical juvenile or in the precocious (late infantile) form. This finding appears to be the most important diagnostic criterion for differentiating the Batten from the Tay–Sachs' type, (TINGEY *et al.*, 1958).

Nor has any increase in neuraminic acid or hexosamine been demonstrated in cases of gargoylism. That such an increase may occur in some cases has been noted by BRANTE (1957) and by KLENK (1955), but the actual figures are not quoted. On the other hand, the figures obtained by BRANTE (1951), i.e. 1·9 per cent ganglioside

in the cortex and 0·15 in the white matter, would not be considered abnormal by modern standards. It is possible that the ganglioside content in gargoylism is variable, particularly if the derangement of glycolipids is only secondary to a primary error in polysaccharide metabolism (BRANTE, 1952). Furthermore, the possibility cannot be ruled out that some of the stored substances are unduly sensitive to formalin fixation, even although BRANTE (1949) showed that the glycolipids of the normal brain are not significantly affected by the fixative. Further work on unfixed material would therefore be desirable.

In the leucodystrophies, irrespective of type, an increase may be found both in total hexosamine values (CUMINGS, 1957) and in the lipid hexosamine fraction (EDGAR, 1957). Our results agree closely with the findings of these authors. It may be pointed out that all hexosamine values are expressed per 100 g of dry tissue, a method preferable to calculation on a wet basis as it is less liable to artefact. Figures thus obtained may, however, imply only a relative and not an absolute increase. A reduction in total solids associated with a relative increase in the water content of the white matter is a constant feature of all demyelinating diseases. It is therefore essential to demonstrate that values are increased both on a wet and on a dry basis before it can be accepted that a substance is present in excess. If the hexosamine values are recalculated to represent the content per 100 g of fresh tissue, the values obtained do not exceed those found in normal controls. It would therefore be more appropriate to speak of preservation of the normal hexosamine levels in the leucodystrophies, rather than of an excess of this substance.

The estimation of total or lipid hexosamine does not throw any light on the nature of the abnormal substances which may have accumulated in the various forms of leucodystrophy. The only observation which may be of some importance is the high value of the water-soluble fraction in relation to the total hexosamine in the case of metachromatic leucodystrophy. This suggests an abnormality of mucopolysaccharide metabolism, and this aspect of the problem is being investigated further.

SUMMARY

(1) Results are given of the estimation of lipid galactose, neuraminic acid and various hexosamine fractions in representative cases of cerebral lipidoses and leucodystrophies.

(2) The methods employed are discussed with emphasis on the inherent weaknesses of calculation methods.

(3) A substantial increase both in neuraminic acid and in all hexosamine fractions was found in Tay–Sachs' disease both in the cortex and the white matter. A moderate increase in these substances was found in the white matter of a case of Niemann–Pick's disease.

(4) No significant abnormality was found in cases of the Batten type of amaurotic idiocy in various age groups, either in fresh or in fixed material. Similar results were obtained on formalin fixed material from two cases of gargoylism.

(5) Cases of leucodystrophy showed increase in hexosamine fractions in the white matter. This is thought to be only relative and due to the reduction of myelin lipids.

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STRUCTURE-ACTIVITY DETERMINANTS OF PHARMACOLOGICAL EFFECTS OF AMINO ACIDS AND RELATED COMPOUNDS ON CENTRAL SYNAPSES

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INTRODUCTION

ANALYSIS of the specific synaptic actions of amino acids and related compounds makes available a new series of drugs for a combined electrophysiological and neuropharmacological attack upon the neurochemical nature of synaptic electrogenic mechanisms in brain. Ranging from very simple to very elaborate, the ordered wealth of structural complexities in which the substances may be presented should make it possible to uncover rules that govern the effects of chemical agents upon the specific varieties of synaptic activity. Furthermore, the natural occurrence of many of the compounds, e.g. γ-aminobutyric acid (GABA) (AWAPARA, LANDUA, FUERST and SEALE, 1950; ROBERTS and FRANKEL, 1950; UDENFRIEND, 1950); γ-guanidinobutyric acid (PISANO, MITOMA and UDENFRIEND, 1957); thiamin and pantothenic acid, suggests that in normal states or in the pathological, or in both, these substances may affect central nervous functioning by pharmacological actions on synaptic surfaces as contrasted with their roles in intermediary metabolism (GRUNDFEST and PURPURA, 1957). Some of the implications for both the theoretical and the clinical aspects of these matters, as well as the detailed evidence for the conclusions of the preliminary reports (PURPURA, GIRADO and GRUNDFEST, 1957*a*,*b*; 1958*a*,*b*,*c*) are incorporated in this paper.

The conceptual basis of the work described here rests upon the demonstration (PURPURA and GRUNDFEST, 1956*a*; 1957*a*) that the major electrocortical activity, and specifically the 10–15 msec surface-negative potential evoked in cat brain by various means of activating the cerebral or cerebellar cortex is composed of postsynaptic potentials (p.s.p.'s) of the superficial dendrites. In that case, the responses must conform in electrophysiological and pharmacological properties to those that are characteristic of p.s.p.'s in general (GRUNDFEST, 1957*a*). This conclusion, confirmed in various ways, has proved outstandingly useful in predicting and accounting for experimental data (GRUNDFEST, 1957*a*; 1958*c*; PURPURA, 1957*a*,*b*; 1958; PURPURA GIRADO and GRUNDFEST, 1957*a*,*b*; 1958*a*,*b*,*c*; PURPURA, *et al.*, 1958*a*,*b*; PURPURA and GRUNDFEST, 1956*a*; 1957*a*; PURPURA, HOUSEPIAN and GRUNDFEST, 1957, 1958; and others in preparation). Those aspects relevant to the present purposes may be summarized briefly.

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The electrocortical activity is a composite of surface-negative responses of depolarizing (excitatory) synapses and surface-positive potentials of hyperpolarizing (inhibitory) synapses in different proportions. The relative amounts of the two algebraically summating potentials depend on the cortical region and on the pathways which activate it. A fortunate circumstance, from the point of view of experimental material, is the relative paucity of hyperpolarizing synapses on superficial dendrites in the cerebellar cortex. This permits differential tests of pharmacological agents which act selectively or non-selectively upon the two varieties of synapses (PURPURA and GRUNDFEST, 1957*a*).

METHODS

Principle. The preceding outline of synaptic organizations in cerebral and cerebellar cortex indicates the analytical method which will be used in the present paper. The data to be described involve only synapse inactivators (GRUNDFEST, 1957*b*) which can produce three types of effects (GRUNDFEST, 1957*a*). (i) Non-specific blockade of both varieties of synapses, such as is produced by D-tubocurarine (DTC), results in elimination of evoked responses or 'spontaneous' electrical activity from the cerebellar as well as cerebral cortex (PURPURA and GRUNDFEST, 1956*a*). (ii) Selective blockade of inhibitory, hyperpolarizing synapses then should, and does, lead to augmentation of the surface-negative response of the cerebral cortex. Loss of inhibitory braking upon excitatory activity leads to 'spiking' and convulsant electrical activity such as occurs with strychnine (PURPURA and GRUNDFEST, 1956*a,b*; 1957*a,b*). However, the selective inactivators of inhibitory synapses should not, and do not (PURPURA and GRUNDFEST, 1957*a*; PURPURA, GIRADO and GRUNDFEST, 1957*b*, 1958*a*) have direct action upon superficial dendritic responses of cerebellar cortex. (iii) Selective blockade of depolarizing excitatory synapses should (and does) lead to unmasking of the surface-positive component in the responses of the cerebral cortex. Since the cerebellar cortex develops little surface positivity in the responses evoked by a local cortical stimulus, abolition of the cerebellar surface negativity results in loss of its response.

These differential effects of pharmacological agents upon cerebral and cerebellar electrocortical responses, predictable on the basis of other data (PURPURA and GRUNDFEST, 1957*a*) were first disclosed by the use of the selectively acting amino acids (PURPURA, GIRADO and GRUNDFEST, 1957*a,b*). The results described in the foregoing papers are illustrated in Figs. 1 and 2.

Preparation. Succinylcholine-paralysed cat preparations, made as described earlier (PURPURA and GRUNDFEST, 1956*a*) were used chiefly. However, among the more than 200 experiments in the course of this work some animals received nembutal (pentobarbital sodium) in various doses. Most frequently, the activity tested was the response evoked in the exposed cerebral or cerebellar cortex by brief (0·1–0·3 msec) electrical pulses applied to the same cortical area. In many experiments, however, other pathways for activating the cortex were also employed.

Surface stimuli were delivered through a pair of 100 μ Teflon-insulated silver wires laid bare at their cross-sections. Two or more independently controlled, sweep-synchronized stimulating channels were available as required. Stimulus strength proved to be an important factor in evaluating the selective mode of action of some of the ω-amino acids tested. Ordinarily, stimuli were carefully selected so that they did not generate significant negativity in the cortical depths by exciting elements under the surface in addition to superficial cortical dendrites. Under these conditions surface polarity reversals of potential produced by selectively acting ω-amino acids were readily differentiated from effects observed with non-specific depressants (KCl, topical pentobarbital, etc.) in a variety of ways (to be published).

Recording from the cortical surfaces was with a monopolar silver ball electrode, differentially against a remote lead. Two recording channels were simultaneously registered on a dual beam oscillograph. In some experiments sub-surface recordings were also made, either with microelectrodes (electropolished stainless steel or saline-filled microcapillaries) or with 100 μ insulated silver wires. In others, a pair of 100 μ insulated silver wires recorded in the pyramidal tract activity initiated by a cortical stimulus. Ink-recordings of electrocortical activity with a standard electroencephalograph were obtained in many of the preparations. Behavioural patterns and motor activity were observed in a few unrestrained cats and in a large series of albino mice and new born rats after intraperitoneal injections of amino acids in quantities up to 1 g/kg.

Application of drugs. The amino acids and related synaptically active compounds do not have conspicuous effects upon intracarotid, intravenous or intraperitoneal injections into cats, and when given by the latter route to mice, even enormous quantities (500 mg/kg), are without obvious central action. This is presumably ascribable to the effectiveness of the blood–brain barrier, since local destruction of this barrier in cats (PURPURA *et al.*, 1958*a*,*b*) permits the intravenously injected synaptic agents to produce actions localized to the site of the barrier lesion. Effects could then be obtained by injecting 8 mg/kg of GABA, as will be described below. Injection of the compounds into the substance of the brain proved unsatisfactory, the synaptic actions being minimal while the resultant damage was apt to be large.

In one series of experiments the compounds were applied intraventricularly by means of a cannula situated in the lateral ventricle. Another, placed in the cisterna magna permitted flushing the ventricular system rapidly after administration of the various buffered amino acids. Substances to be tested were slowly introduced in 0·5 ml quantities after prior removal of an equal volume of cerebrospinal fluid. Electrocortical activity was monitored during and for long periods after administration.

Of the several ways of topical administration that were tried in preparations with blood–brain barrier intact, one was adopted for its simplicity and for the reproducibility of the results. The substances were made up in 0·1 to 1·0% concentrations in Ringer's solution, buffered at pH 7·4. It was desirable to use organic buffers to avoid the addition of more inorganic ions. However, some of the commercially available organic buffers proved to contain synaptically active compounds. 2-Amino-2-hydroxymethylpropane-1:3-diol (TRIS) was found to be synaptically inert.

For the test, from two to five drops of the solution were applied to a cortical region, using a hypodermic syringe and needle. If an effect was produced rapidly, as in the case of the synaptically active drugs, a second application was made after an observation period of 1–2 min. After another period of observation the cortex was flushed with Ringer's solution. If further tests were to be done upon the same cortical site, repeated rinsing for 15–30 min was performed to insure that there was no remnant of the solution used earlier. When a series of synaptically active substances were thus tested, their order was varied in different experiments. The substances listed in Table 1 as synaptically inert were so judged on the basis of the absence of effects of three or more applications over a period of 10 min or more. The synaptically active drugs, all of which produced effects within a few seconds after application in solutions of 0·1% to 1% concentrations, were thus clearly distinguishable from the synaptically inert compounds.

Avoidance, or recognition of interactions was one of two major precautions. The other was that the mechanical disturbance of applying fluid to the cortex did not change contacts either at the stimulating or at the recording electrodes. Prior to beginning a test, the cortex was repeatedly flushed with Ringer's solution, and only those electrode arrangements were used in which the testing responses were not affected by the procedure. Another check made in all cases was the reversibility of the drug action when the drug was rinsed away. Since both onset of the drug action and its disappearance were rapid, errors were easily noted.

Effective concentrations. The compounds that are listed as synaptic agents in Table 1 had identical actions when applied in different concentrations, except that effects were produced more rapidly when these substances were applied in higher concentrations (1%). In a few experiments 5% solutions were also applied. The effects were essentially the same as those produced by the 1% solutions.

Only approximate statements regarding the effective concentrations of the drug on the population of synapses can be made at present. Most of the applied solution ran off the convexity of the cortical surface. What remained was diluted by the fluid layer on this surface. The rates of penetration of the compounds are not known, and the concentrations of the substances in their final action are also dependent upon local anatomical factors; amount of the circulation, density of the dendrites, their depth below the surface and other conditions.

Obviously, however, the amount of the drug acting upon the synapses cannot have been very large, as may be seen from an approximate calculation designed only to give the order of magnitudes involved. In two to three drops of a 1% solution, the amount of the amino acid drug is about 2 mg. Assuming that as much as 1 per cent of the solution reaches the sub-surface sites with which the drug reacts, this would make about 20 μg of the substance available for synaptic action. This amount compares with the quantities of drug required in situations where quantitative measurements are possible. Thus, eel electroplaques require to be bathed in solutions with minimal concentration of 25 μg/ml of eserine, or 50 μg/ml of DTC for blockade of their synapses (GRUNDFEST, 1957*c*).

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In some experiments the substances to be tested were applied on squares of filter paper. Continued application of 10^{-5} w/v of GABA caused effects similar to, but less pronounced and developing more slowly than those produced by two or three drops of the 1% solution. The minimal concentration of GABA that caused an effect was 7 μg/mm^2 of cortical surface under the filter paper. The effectiveness of as little as 8 mg/kg of GABA upon intravenous injection in preparations with altered blood–brain barrier permeability also indicates that the compound acts in low concentration (PURPURA *et al.*, 1958*a*).*

RESULTS

Types of Surface Electrocortical Effects Produced by the Synaptically Active Amino Acids

Effects of the aliphatic ω-amino acids C_2 *to* C_5. Within 1–2 sec after applying one of these compounds to the cortical surface, and particularly GABA (C_4), the electrocortical activity evoked in both the cerebral and cerebellar cortex changes markedly (Fig. 1). The cerebellar response decreases and disappears, so that the drug creates

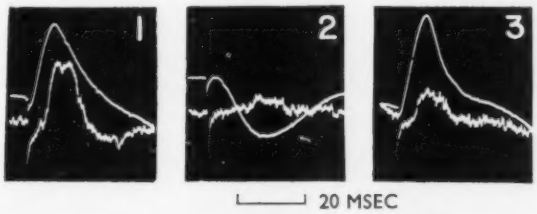

FIG. 1.—Differential effects of GABA on cerebral and cerebellar superficial dendritic postsynaptic responses. (1) Oscillograph records of the surface-negative responses evoked in cerebral cortex (upper trace) and cerebellar (lower trace) by simultaneously delivered, independent, brief stimuli to each surface. (2) GABA (five drops, 10^{-3} w/v) was applied to each cortical surface, causing reversal of the cerebral response and elimination of the cerebellar. (3) Both cortical responses recovering 2 min after flushing the surfaces with Ringer's solution, the cerebellar not as rapidly as the cerebral. Time, 20 msec.

an essentially unresponsive cortex. This is not the case for the cerebral response. The surface negativity of the latter is inverted to a large surface-positive activity. The effects of the drug are reversible.

The actions produced by GABA can be duplicated by its homologues of carbon chain lengths C_2 to C_5 (Fig. 2). β-Alanine (C_3) is almost as effective as is GABA in producing an inversion of the cerebral evoked response, but acts more slowly. Glycine (C_2) and δ-aminovaleric acid (C_5) are less effective, some initial surface negativity remaining and the surface positivity being smaller. Upon rinsing away the amino acid, return of negativity entails a phase of augmented responses (Fig. 2C). The cerebellar effects of the C_2–C_5 compounds are like those shown for GABA in Fig. 1.

Effects of longer-chain ω-amino acids. The different action on cerebral and cerebellar cortex seen in Fig. 1 are elucidated (Fig. 3) by the effects produced at the two structures by ε-aminocaproic acid (C_6) and ω-aminocaprylic acid (C_8). Both compounds, as well as their C_9 homologue (ω-aminononanoic acid), augment the

* A recent note which reports effects that are ascribed to intracarotid injections of GABA (MARRAZZI *et al.*, 1958) criticizes topical applications as 'highly abnormal.' It seems pointless at this stage of scientific development to debate the definition of 'normal' or 'physiological' conditions during an experiment. However, with respect to the study of central actions of drugs it is the apparently more 'physiological' route which is subject to the blood–brain barrier. The experiments specifically designed to circumvent the latter (PURPURA *et al.*, 1958*a*,*b*) fully confirm the results obtained by topical administration of the ω-amino acids.

responses evoked from cerebral cortex, but produce no significant effect on cerebellar activity.

Interpretation of synaptic effects of ω-amino acids. The differential tests shown in Figs. 1 and 3 point to the mechanisms of synaptic action of the respective causative agents. Like DTC in heparinized preparations (PURPURA and GRUNDFEST, 1956*a*,

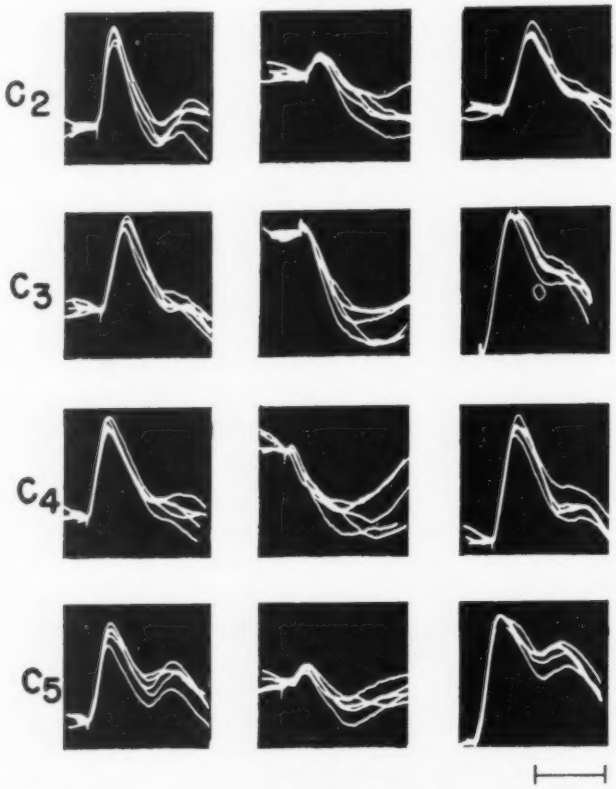

FIG. 2.—Blockade of depolarizing, surface-negative dendritic p.s.p.'s in cerebral cortex by ω-amino acids. All tests were performed on the same preparation. About 0·3 ml each of glycine (C_2), β-alanine (C_3), γ-aminobutyric acid (C_4) and δ-aminovaleric acid (C_5) were applied to the cortex in 1% buffered solution at 20–30 min intervals. (*Left column*)—five superimposed consecutive records of the dendritic p.s.p. evoked immediately before applying the amino acid. (*Middle column*)—2 min after the application. The C_2 and C_5 compounds produced less surface positivity than did the C_3 and C_4 ω-amino acids and a remnant of surface negativity is seen in both cases. (*Right column*)—5 min after flushing the cortex with warm Ringer's solution. The temporary augmentation of the dendritic surface negativity after the C_3 to C_5 compounds is characteristic. Time, 20 msec.

1957*a*), the C_6, C_8 and C_9 ω-amino acids augment the cerebral activity and leave unaffected the cerebellar. The interpretation valid for that case appears also to apply here. These compounds are to be considered as blockaders of surface-positive, hyperpolarizing, inhibitory synaptic activity, which is prominent in the directly evoked dendritic response of the cerebral cortex, but not of the cerebellar. Selective blockade of this activity in the cerebral cortex thus would augment the evoked surface-negative response. Since the corresponding response of the cerebellar cortex does not

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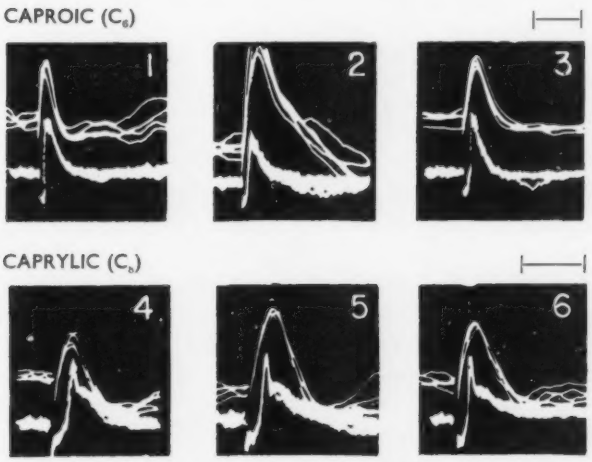

FIG. 3.—Differential actions of caproic (C_6) and caprylic (C_8) ω-amino acids on cerebral and cerebellar cortex. Records for the two compounds taken from two different experiments. Direct comparison of the augmentation produced by C_6 and C_8 on the evoked p.s.p. of cerebral cortex therefore cannot be made: (1, 4) controls; (2, 5) 1 min after application of agents to cerebral (upper trace) and cerebellar cortex, a striking augmentation of responsiveness occurs in cerebral but not in cerebellar cortex; (3, 6) 20 min after frequent rinsing of the cortical sites with warm Ringer's solution. Time, 20 msec.

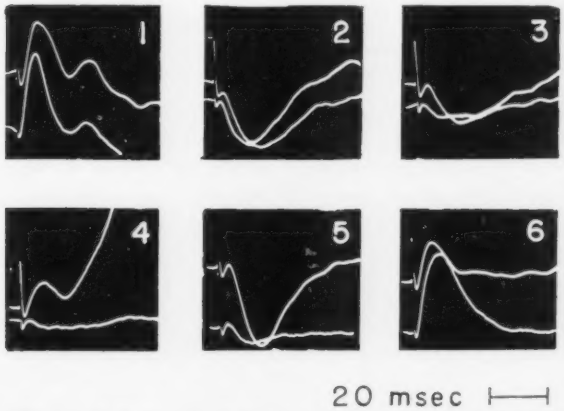

FIG. 4.—Different modes of interaction of GABA with picrotoxin and strychnine. Simultaneously recorded responses to independent stimulations of homologous points of right (upper trace) and left (lower trace) anterior suprasylvian gyrus, the cerebral hemispheres having been disconnected by callosal section: (1) initial dendritic postsynaptic potentials; (2) 30 sec after application of GABA (three drops, 10^{-2} w/v) to each side. Picrotoxin (two drops of 3×10^{-3} w/v solution) was then applied to right cortex and strychnine sulphate (two drops of 5×10^{-3} w/v) to the left; (3) 30 sec later; (4) 1 min later, after another application of the convulsants; (5) 2 min later and 30 sec after another application of GABA (whereas the effects of the strychninized (left) cortex were minimal, marked antagonism to picrotoxin in seen); (6) 15 min after repeated washing of the cortex with Ringer's solution. Recovery of previously inactive strychninized side is the more rapid, probably denoting persistent blockade of inhibitory synapses.

include a conspicuous amount of hyperpolarizing p.s.p.'s these drugs do not have a synaptic substrate and are without effect on the evoked potential.

The explanation of the effects of the C_2–C_5 compounds is complementary to and consistent with that presented above. To various degrees the C_2–C_5 substances are selective blockaders of depolarizing, excitatory surface-negative p.s.p.'s. Removal of this activity without block of the hyperpolarizing synapses should result in a surface-positive response in the cerebral cortex. However, there would be little or no positivity developed in the cerebellar cortex and a reversal of its potential thus would not develop. Both responses behave in accordance with this expectation (Figs. 1, 2).

Pharmacological confirmation of the action of GABA. Further evidence in support of this interpretation is provided by experiments such as those of Fig. 4. The cerebral hemispheres were separated functionally by callosal section. Testing stimuli were applied to homologous regions of both sides. When reversal was achieved in both by GABA (2) picrotoxin was applied to one site and strychnine to the other. The first substance, like Metrazol (TOMAN, 1955) an activator of excitatory synapses, behaves as a competitive antagonist of GABA. Unlike picrotoxin (ECCLES, 1957) strychnine, a selective inactivator of inhibitory synapses (PURPURA and GRUNDFEST, 1956*a*,*b*, 1957*a*,*b*), exerts inactivating effects which therefore are complementary to those of GABA upon the depolarizing synapses and eliminate all dendritic activity (3, 4). The effects of both substances are reversed by additional GABA (5), although the kinetics of the interactions appear to be different. Rinsing both cortical areas restores the initial activity (6), again with some manifestations of rate differences.

Effects of ω-amino acids on EEG. These are shown in Fig. 5. Topical applications of C_3 and C_4 produce some augmentation of the amplitudes and slowing of the frequency of the EEG. On the other hand, the C_6, C_8 and C_9 compounds, and particularly the latter, cause spiking in the cerebral cortex. They are about as effective as is topically applied strychnine, which also exerts this action by virtue of its selective blockade of inhibitory synapses (PURPURA and GRUNDFEST, 1956*a*, 1957*a*,*b*), and which like the C_6, C_8 and C_9 ω-amino acids does not evoke paroxysmal activity in the cerebellar cortex when applied topically.*

Sites of action of ω-amino acids. (a) *Blood–brain barrier.* Intravenous injections of the ω-amino acids in cats or peritoneal injections in mice, in which relative concentrations could be made very high, have no or only slight effects upon behaviour or upon electrocortical activity, whether spontaneous or evoked. This absence of action is not surprising in view of the reported lack of GABA uptake by brain *in vivo* (ROBERTS and BAXTER, 1957). When the barrier is eliminated (PURPURA *et al.*, 1958*a*,*b*), intravenous injections of the ω-amino acids have effects both on the EEG and on the evoked potentials, but only in areas of increased vascular permeability (Fig. 6).

(b) *Axo-dendritic synapses.* On the basis of these findings it is therefore not

* Extension of earlier studies (PURPURA and GRUNDFEST, 1957*a*) indicates that topically applied strychnine affects cerebellar cortex differentially depending on the mode of its activation. In this respect strychnine and the C_6 and C_8 ω-amino acids have qualitatively similar actions. All are inert on surface p.s.p.'s evoked by direct cerebellar stimulation and do not initiate spontaneous paroxysmal activity. On the other hand, all augment late negative components, but abolish or reduce the prior positive components of some evoked cerebral–cerebellar responses (unpublished data). Although hyperpolarizing electrogenesis is not generated on dendritic elements responsible for the direct cerebellar response, inhibitory synaptic activity results from afferent bombardment of cerebellar neurones by some pathways. Selective engagement of inhibitory neurones in cerebellar cortex, particularly in paramedian lobule, is strongly dependent on the afferent source of activity (JANSEN, 1957; and unpublished work from this laboratory).

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surprising that the ω-amino acids act principally in topical applications, less effectively by the intraventricular route and have relatively insignificant actions by intracarotid injection. However, additional data restrict their sites of effectiveness even further and provide a new pharmacological specification of synaptic structures. GABA was applied (Fig. 7) to a cortical region, the activity of which was recorded both from the

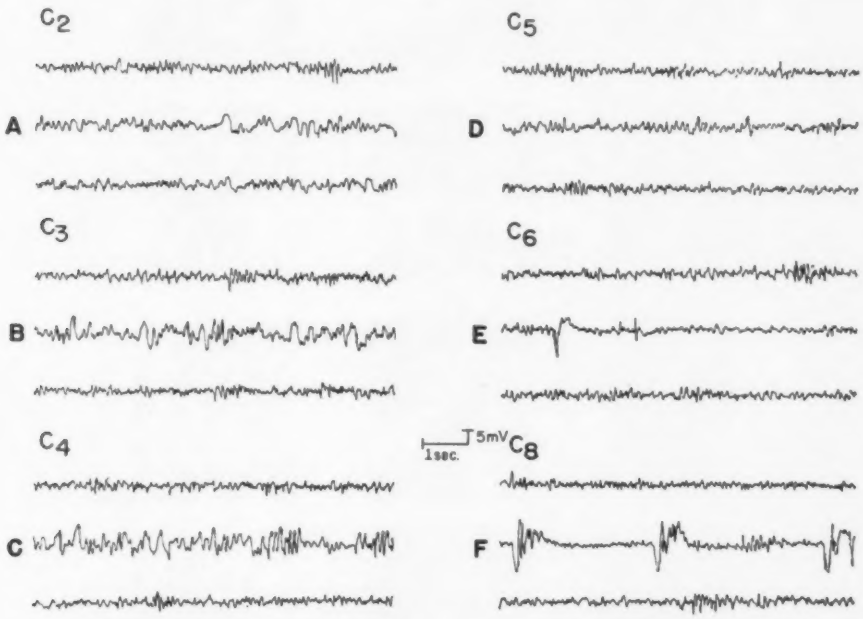

FIG. 5.—Effects of topical application of ω-amino acids on cerebral electrocortical activity. Same experiments as in Fig. 2. Bipolar leads, one on the anterior suprasylvian, the other on posterior sigmoid gryus. In each set (A–F), a different amino acid was tested identified by designations C_2–C_8. The upper record of the set was taken before applying the compound, the middle 2 min after its application, and the lower 5 min after flushing the cortex with warm Ringer's solution. Pronounced slowing and increased amplitude of the activity were induced maximally by the C_3 and C_4 ω-amino acids (B, C). The C_6 compound induced occasional hypersynchronous discharges (E), while the C_8 amino acid caused rhythmical high amplitude discharges and eventually, convulsive activity. In all cases the electrocortical actions were rapidly reversed.

surface and from a sub-surface lead that was located about 0·4 mm deep in an essentially iso-electric region (2). The profound reversal of potential on the surface (3, 4) found no reflection below. Nor did restoration of the initial surface negativity (5) affect the sub-surface lead. Experiments of this type have been carried out with surface responses evoked by various pathways afferent to the cerebral cortex (PURPURA, 1958). The results in all cases (Fig. 8) lead to the conclusion that GABA acts primarily and perhaps solely on the superficial dendritic activity, and does not affect synapses of the cortical depth.

An experiment testing specifically the effects of C_4, C_6 and C_8 on the axo-somatic synapses of cortico-spinal cells is shown in Fig. 9. The directly evoked responses of these cells, and the synaptically induced later repetitive activity were recorded as discharges in the pyramidal tract. The synaptically activated components have been

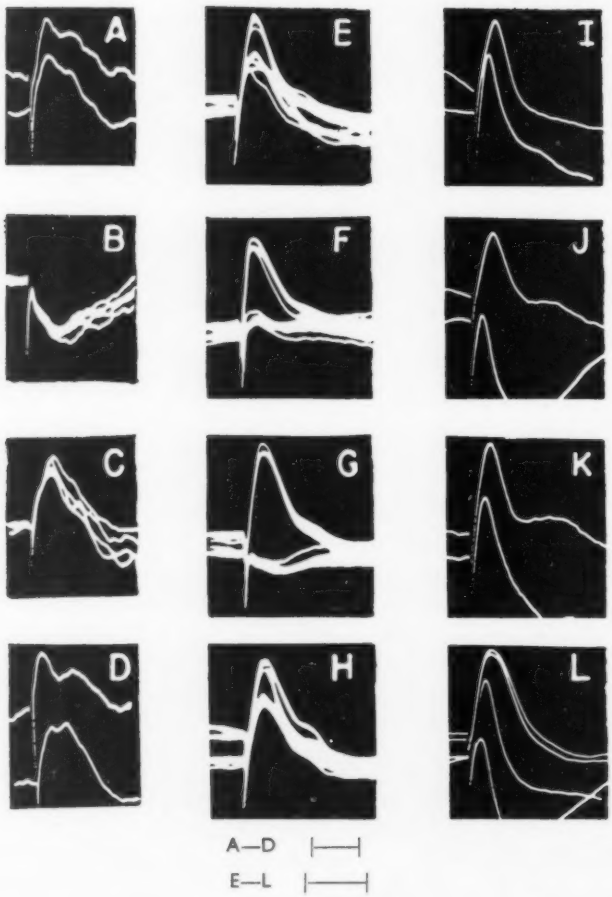

FIG. 6.—Effectiveness of systemically administered GABA in cortical regions of blood–brain barrier loss. (A–D) Ethyl chloride induced barrier lesion on one side, succinylcholine preparation. Upper channel in (A, D) and trace in (B) from site of lesion, delineated in the course of the experiment by staining with Evans' blue dye. Lower channel record in (A, D) and trace in (C) from homologous, opposite, barrier-intact cortex. (A) Simultaneous records before injecting GABA. (B, C) Superimposed consecutive responses from site of lesion (B) and barrier-intact cortex (C), 40–50 sec after GABA, 50 mg/kg i.v. (D) After 1 hr simultaneous traces from both recording sites. (E–H) Barrier lesion induced by chloroform–methanol; preparation under pentobarbital, 15 mg/kg. Simultaneous recordings on two oscillograph channels, superimposed traces. Upper channel records from intact cortex, lower from region of barrier lesion. (E) Before injection. (F) After 8 mg/kg GABA. (G) After 25 mg/kg. (H) After 90 min from (G). (I–K) Ethyl chloride induced barrier lesion: preparation under pentobarbital (15 mg/kg). Simultaneous recordings from intact (upper) and lesion sites (lower). (I) Before and (J) after injection of GABA, 25 mg/kg i.v. The effect on the region of barrier lesion disappeared slowly. (K) Showing recovery after 45 min. (L) Records like those of (I) and (J) superimposed to show ineffectiveness of systemic GABA in barrier-intact cortex (two upper traces) and profound effect in region of barrier-loss (two lower traces). Time calibrations, 20 msec.

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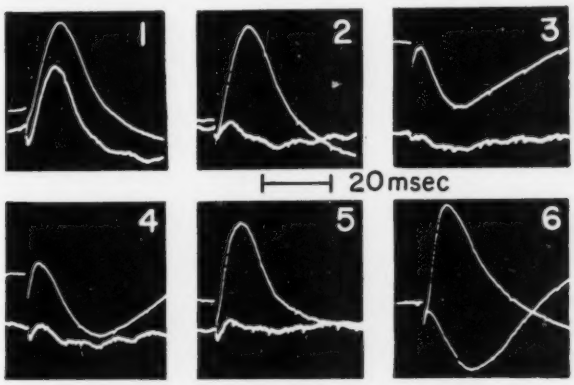

FIG. 7.—Restricted action of GABA on superficial dendritic synapses of cortex. (1–5) Simultaneous recording with large, surface electrode (upper trace) and small (100 μ) probe electrode. The probe was on the surface in (1) and about 0·4 mm below in (2–5). This region was approximately isoelectric with respect to an indifferent lead and remained so despite the large changes in surface potential produced by topical application of GABA (3). Beginning recovery (4) and full recovery after removal of the amino acid (5). The extent of the shift in surface potential is shown in the superimposed records of (6). Time calibration, 20 msec.

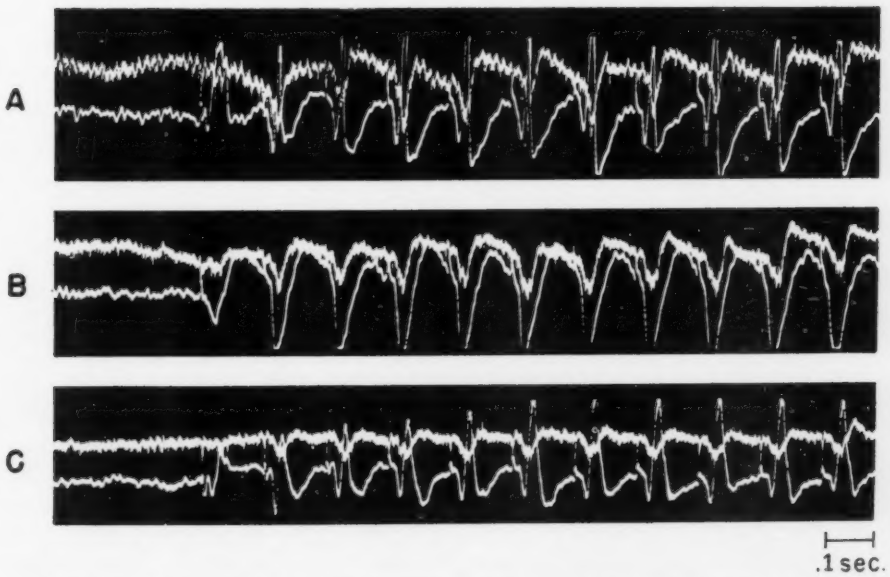

FIG. 8.—Independence of associated cortico-spinal discharges, despite modification of superficial cortical augmenting activity produced by GABA. Simultaneous recordings, upper trace, from electrodes in medullary pyramidal tract, lower from surface of ipsilateral anterior sigmoid gyrus. (A) Beginning of a train of stimuli at 8/sec applied to thalamo-cortical pathway which evokes the initially small, but rapidly augmenting cortical response, characterized by an early positivity and large growth of a surface negativity. Associated with cortical response is a discharge in the pyramidal tract. This trace is marred by a 60 cycle artifact. (B) About 30 sec after applying GABA to the cortex the augmenting responses became predominantly surface-positive, denoting the elimination of surface-negative dendritic p.s.p.'s. The discharges evoked in the pyramidal tract were not affected. (C) About 5 min after rinsing the cortex with Ringer's solution.

shown to be eliminated by DTC (PURPURA and GRUNDFEST, 1956*a*). However, GABA, a powerful blockader of depolarizing synapses, does not alter the pyramidal tract activity. Neither does the blockade of hyperpolarizing synapses by C_6 affect the response. On the other hand, the C_8 compound evokes very considerable after-discharge, as does strychnine (PURPURA and GRUNDFEST, 1956*b*, 1957*b*). The present evidence does not permit a decision as to whether C_8 acts directly upon the axo-somatic inhibitory synapses or indirectly through profound effects upon the dendritic

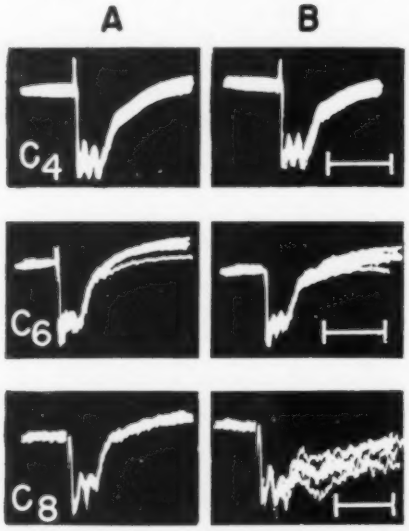

FIG. 9.—Effects of C_4, C_6 and C_8 ω-amino acids on cortically evoked activity in pyramidal tract. Responses recorded at mid-olivary level. (A) Control; (B) 10 min after repeated application of the solutions to motor cortex. Each of the agents tested in a different experiment. Ten superimposed tracings for the C_4, and five superimposed tracings for the C_6 and C_8 series. Significant augmentation in synaptically evoked late responses occurs only with C_8 ω-amino acid. Time, 10 msec.

synapses and cortical spiking. However, it is clear that the strong synaptic actions of opposite kinds produced by C_4 and C_6 do not affect the axo-somatic synapses.

In topical applications, the actions of these compounds might be restricted to axo-dendritic synapses because the substances do not reach the axo-somatic synapses of the Betz cell layer. This is not the case, as is seen from experiments in which the blood–brain barrier was destroyed widely in the pericruciate cortex. Fig. 10 shows the dendritic potentials evoked from such a region and the pyramidal tract activity produced by stimulating the same region. Intravenous GABA reversed the dendritic response, but neither the pyramidal tract activity nor the thalamo-cortical volleys evoked by the strong surface stimuli were affected, even in minor detail. Therefore it must be concluded that the ω-amino acids, in addition to their selectivity for depolarizing or hyperpolarizing synapses, also act preferentially upon axo-dendritic synapses and probably affect the cortical axo-somatic synapses little or not at all as far as can be determined with available techniques.

Use of GABA for analysing synaptic activities. The selective actions of the ω-amino acids in blockading either depolarizing or hyperpolarizing synapses can be employed

to analyse the constituents of various cortical responses. One example is shown in Fig. 11. The cerebral dendritic surface negativity evoked by an antidromic volley is eliminated by GABA, but is not 'reversed.' This indicates that the antidromically activated cortico-spinal cells discharge into a pathway which engages only or chiefly depolarizing synapses of the superficial cerebral dendrites. Failure to generate significant inhibitory synaptic activity on apical dendrites by antidromic cortical stimulation satisfactorily accounts for the striking ineffectiveness of topical strychnine

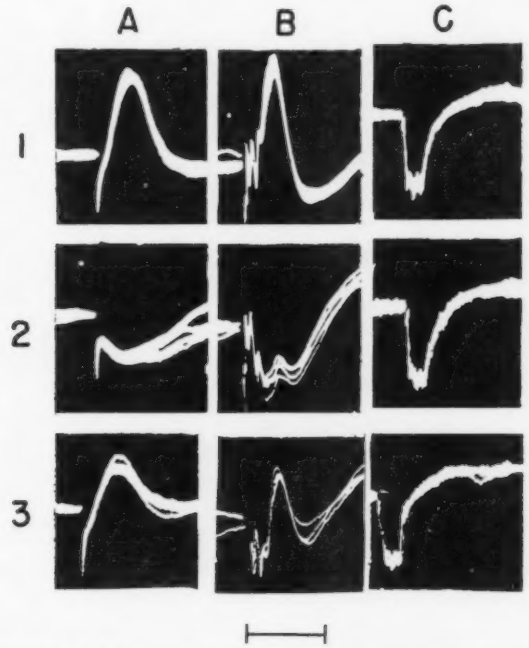

FIG. 10.—Comparison of effects of systemically administered GABA on axo-dendritic and axo-somatic synapses. Anterior and posterior sigmoid gyri of one side had been treated with chloroform–methanol 2 hr prior to recording. Blood–brain barrier lesion verified subsequently by staining with Evans' blue dye. Row (1) before; (2) 1 min after injection of GABA (30 mg/kg i.v.); (3) 1 hr later, beginning of recovery. (A) Response to weak superficial stimulus, recorded from posterior sigmoid gyrus inverted after injection of GABA. (B) A stronger stimulus evoked early spike activity which was not affected when the large dendritic response was reversed by GABA. (C) The same strong cortical stimulus evoked direct and relayed discharges in cortico-spinal tract recorded at mid-olivary level. These were not affected by GABA. Superimposed traces of five consecutive sweeps in each record. Time calibration, 20 msec.

on the surface-negative component of the antidromic response (LANDAU, 1956; PURPURA and GRUNDFEST, 1956*b*, 1957*b*). Pathways deficient in inhibitory synapses provide a substrate for demonstrating the non-specific synapse blocking effects of strychnine (PURPURA and GRUNDFEST, 1957*a*), as well as of some of the amino acid derivatives described below. Further analyses of synaptic pathways that have been made in cat and bullfrog central nervous system with ω-amino acids are described elsewhere (PURPURA, GIRADO and GRUNDFEST, 1958*b*; SIGG and GRUNDFEST, 1958; and to be published).

Synaptic Actions of Other Amino Acids

The tests used in the foregoing were applied to other amino acids and to related compounds. Because of the large number of substances already tested, the data are presented chiefly in tabular form (Table 1), and only the actions of certain selected compounds are described in some detail.

The aliphatic α-homologues of the ω-amino acids. None of the α-amino acids, with the exception of glycine (which belongs in either category), had synaptic effects (Table 1). Only three diamino compounds of this series were tested. As seen from

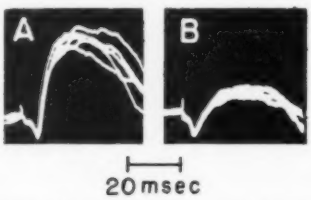

FIG. 11.—Action of GABA on antidromic response evoked at cortical surface by stimulating medullary pyramids. The large dendritic negativity (A) was eliminated, but not inverted (B) by applying GABA to the recording surface in lateral margin of cruciate sulcus. A positive component which probably represents activity of cortical interneurones also was not affected. Five responses superimposed in each record.

Table 1 the synaptic action of the corresponding ω-compound was eliminated or greatly weakened by the extra amino group.

Other amino acids. Structurally these compounds represent so heterogeneous a collection that the effects of some, which are synaptically active, will be described below in relation to structural factors. The synaptically active and inactive compounds tested are listed in Table 1.

Experimental Analysis of Amino Acid Action in Terms of Structural Factors

The sharp distinction between the synaptically active ω-amino acids and their synaptically inert α-homologues suggests that steric and electrostatic factors may play a part in the determination of synaptic actions. The ω-amino acids, in a first approximation, can be partitioned into three compartments, comprised of the amino terminal group, the carboxylic terminal group and the aliphatic chain linking the two. The comparative data on the effects of different length ω-amino acids indicate that not only must variations in degree of synaptic action be expected, but also that the quality of the synaptic effects may be determined by structural factors. In this respect the amino acids and their related compounds present an almost unrivalled wealth of variation. Thus far, however, only a small part of the available material has been tapped, and is presented in this section.

Role of the aliphatic chain length

(a) *Guanidino acids.* (i) The ω-guanidino acids $HO-\overset{\overset{O}{\|}}{C}-(CH_2)_{(n-1)}-\overset{\overset{H}{|}}{N}-\overset{\overset{NH}{\|}}{C}-NH_2$

provide a series of compounds which closely parallel in structure the ω-amino acids. Tests of these substances, using the methods described earlier, have proved highly

instructive. Guanidinoacetic acid ($n = 2$) blocks depolarizing dendritic synapses (Fig. 12) about as powerfully as does GABA (C_4) and unlike the weakly acting glycine (C_2). The β-guanidinopropionic acid ($n = 3$), is also a powerful inactivator of depolarizing synapses. On the other hand, the $n = 4$ and higher guanidino acids block hyperpolarizing synapses, as C_6, C_8 and C_9 of the ω-amino acids do. Thus it

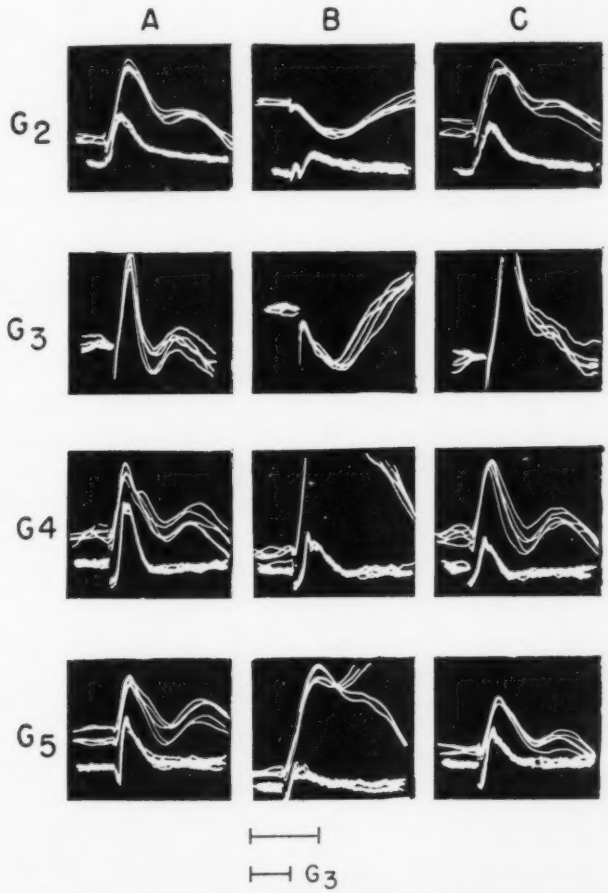

FIG. 12.—Differential effects of ω-guanidino acids on evoked response of cerebral and cerebellar cortex. G_2–G_5 denote respectively the acetic, propionic, butyric and valeric ω-guanidino acids. All but G_3 recorded from one experiment with simultaneous observation of effects on cerebral (upper trace) and cerebellar responses. Only cerebral records shown for G_3 which was also taken with a slower time base. (A) before; (B) about 30 sec after topical application; (C) about 10 min after rinsing the cortical surface. Five consecutive responses superimposed in each record. Time calibrations, 20 msec.

appears that the guanidino acids of carbon length n behave like the ω-amino acids of carbon lengths $n + 2$. This implies that the link $-\overset{|}{\underset{|}{N}}-\overset{\|}{\underset{NH}{C}}-$ which ties the NH_2 terminal to the aliphatic chain is equivalent to two CH_2 links of that chain.

TABLE 1.—AMINO ACIDS AND RELATED COMPOUNDS

	Synaptic effect block EPSP	IPSP	None	Derivatives or congeners	Synaptic effect block EPSP	IPSP	None
(A) *ω-Amino acids*							
C_2 Glycine	++			Sarcosine (methylglycine)			○
C_3 β-Alanine	++++			(1) α-Alanine			○
				(2) α-CH_3-β-alanine			○
				(3) Carnosine-(β-alanyl-L-histidine)	++		
				(4) Anserine (β-alanyl-L-methyl-L-histidine)			○
				(5) Pantothenic acid (pantoyl-β-alanine)		++	
C_4 γ-Aminobutyric acid (GABA)	++++			(1) γ-Butyrolactam			○
				(2) α-Aminobutyric			○
				(3) α-Amino*iso*butyric			○
				(4) 2:4-Diaminobutyric	+		
				(5) γ-Amino-β-OH butyric	++++		
				(6) γ-Aminobutyramide			○
				(7) γ-Dimethylaminobutyric	+		
				(8) γ-Aminobutyrobetaine		+++	
				(9) Carnitine (γ-amino-β-OH butyrobetaine)		+++	
				(10) Acetylcarnitine			○
				(11) Threonine (α-amino-β-OH butyric)			○
				(12) α-Phenyl GABA			○
C_5 δ-Aminovaleric	++			(1) Ornithine (2:5-diamino)			○
				(2) Norvaline (α-amino)			○
				(3) Valine (α-amino*iso*-)			○
				(4) δ-Amino-α-methylvaleric			○
C_6 ε-Aminocaproic		+++		(1) ε-Caprolactam		++++	
				(2) Leucine (α-amino*iso*-)			○
				(3) Norleucine (α-amino)			○
				(4) Lysine (2:6-diamino)		+	
C_8 ω-Aminocaprylic		++++					

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C_9 ω-Aminononanoic	++++		
(B) *ω-Guanidino acids*			
C_2 Guanidinoacetic	++++	(1) Creatine (*N*-methylguanidinoacetic)	+++
		(2) Creatinine	+++
C_3 Guanidinopropionic	++++	(1) α-Guanidinopropionic	+
C_4 Guanidinobutyric	++ ++++	(1) α-Guanidinobutyric	++
		(2) Ureidobutyric	○
C_5 Valeric	++ ++++	(1) Arginine (α-amino)	○
		(2) Canavanine (α-amino-oxy-)	○
		(3) Agmatine sulphate	○
C_9 Guanidinononanoic	++++		
(C) *Dicarboxylic acids*	Blockade of all p.s.p.'s slowly reversible spreading depression	(1) *N*-acetylaspartic	○
Aspartic		(2) Asparagine	Like aspartic
Glutamic		(1) Glutamine	○
α-Aminopimelic		(1) L-Guanidinoglutamic	Same as glutamic, slower action
(D) *Sulphur containing*			
Cysteine	+	Taurine	○
Methionine	○	Methionine sulphoxide	○
(E) *Miscellaneous*			
Phenylalanine	○		
Tryptophan	○		
Histidine	○		
Proline	○		

However the equivalence, though striking, is not complete. While the C_6 and C_8 ω-amino acids have almost no effect upon direct responses of cerebellar cortex (Fig. 3) the C_4 and C_5 guanidino acids depress the response markedly (Fig. 12) at the same time that they augment the evoked cerebral activity. This indicates that the guanidino acids are not as selective as are the ω-amino acids with respect to their synaptic substrate, and blockade depolarizing synapses as well as the hyperpolarizing.

(ii) Two α-guanidino acids were tested (propionic and butyric, Table 1). Unlike the analogous α-amino acids they exerted synaptic action. Whereas β-guanidinopropionic acid blocked depolarizing synapses of cortical dendrites, the α-homologue blocked the hyperpolarizing in cerebral cortex, but did not affect cerebellar responses.

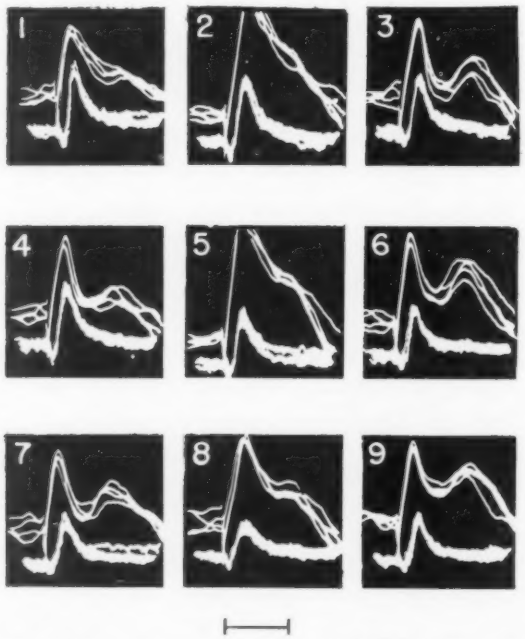

FIG. 13.—Alteration of synaptic properties of ω-guanidino acids by substitutions. Modification of guanidinoacetic acid (G_2) to creatine (1–3) or creatinine (4–6) produces substances that are selective blockaders of inhibitory synapses. Arginine (7–9), derived from δ-guanidinovaleric acid, is a weakly acting agent. Dual traces in all records show the effects on cerebral (upper) and cerebellar (lower) dendritic activity. Four superimposed responses in each record. Time, 20 msec.

α-Guanidinobutyric acid exerted a weaker effect on evoked responses than γ-guanidinobutyric acid. Although both α-guanidino acids may be viewed as substituted guanidinoacetic acid, reversal of the synaptic action of the latter indicates that methyl or ethyl substitution in the aliphatic chain of ω-amino and ω-guanidino acids can exert profound effects on synaptic specificity as indicated below.

(b) *Substitutions in the chain.* (i) The naturally occurring creatine and creatinine are *N*-methylated derivatives of guanidinoacetic acid. This methyl substitution produced compounds that are also synaptically active, but chiefly in the opposite manner from the parent substance (Fig. 13).

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(ii) Weakening of synaptic action by introducing a side chain is particularly notable in the ω-amino acids. Thus, β-amino*iso*butyric acid (or α-methyl-β-alanine) is inactive, whereas β-alanine is about as powerful a synaptic agent as is GABA. Methylglycine (sarcosine) and α-phenyl GABA are synaptically inert as is δ-amino-α-methylvaleric acid.

(iii) However, β-OH GABA is about as potent a synaptic agent as is GABA, confirming the findings of HAYASHI (1956) and MCLENNAN (1957). *o*-Acetyl substitution in carnitine renders the latter synaptically inert.

(iv) As is the case with some ω-amino acids, the insertion of an α-amino group into δ-guanidinovaleric acid resulted in a synaptically inert compound, arginine (Fig. 13).

(v) The change from a guanidino to a ureido configuration has been tested only in the butyric compound. Ureidobutyric acid is without synaptic action. Probably, however, the effect is not merely in the alteration of the chain. Substitution of =O for =NH close to the terminal $—NH_2$ probably affects the electrostatic charge distribution of the molecule.

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Role of the $—NH_2$ *terminal group*

(a) *Betaines.* Two compounds of this type have been tested and both exert strong synaptic action. One, the γ-aminobutyrobetaine is a simple substitution product, while the second (carnitine) has a further substitution of —OH for —H in the β-carbon. Both are relatively selective blockaders of inhibitory synapses. Thus it seems that quaternization with consequent strengthening of the positive charge beyond that in the $—NH_2$ terminal group converts the selective blockaders of depolarizing synapses, GABA and β-OH GABA to substances that act selectively on the hyperpolarizing synapses. In contrast to the effects produced by exhaustive methylation of the terminal nitrogen, dimethyl substitution in the terminal $—NH_2$ of GABA results in a relatively inert compound.

(b) *Lactams.* Cyclization of GABA to form the γ-butyrolactam (α-pyrrolidinone) converts the powerfully acting synaptic drug to an inactive substance. On the other hand the synaptic action of ε-amino caproic acid is markedly enhanced in the ε-caprolactam. In addition this substance is a powerful convulsant (GOLDBLATT *et al.*, 1954) intravenously administered. Therefore, ε-caprolactam must be presumed to penetrate the blood–brain barrier. Creatinine is also a cyclic product that is synaptically active.

(c) The substitution of $—CH_3$ in the $—NH_2$ radical has been tested in sarcosine (*N*-methylglycine). This substance is synaptically inert. It will be recalled that methyl substitution in the second —N— of guanidinoacetic acid converts the powerful and selective blockader of depolarizing p.s.p.'s to creatine, which acts primarily on the hyperpolarizing synapses (Fig. 13). The relation between ω- and α-guanidino acids has been previously mentioned.

(d) Substitution of a complex aliphatic compound (pantoic acid) for one of the amino hydrogens of β-alanine also converts this selective blockader of depolarizing synapse to an inactivator of the hyperpolarization. The substance is pantothenic acid (pantoyl-β-alanine). Like carnitine and thiamin it belongs to the B group of vitamins.

Role of the —COOH *terminal*

The carboxyl group appears to be important for the synaptic action of the ω-amino acids, but like the $—NH_2$ terminal it can under certain conditions be substituted.

(a) Conversion of GABA to the amide results in a synaptically inert compound.

(b) Substitution of $—SO_3H$ for the —COOH of β-alanine also leads to a synaptically inert substance, taurine.

(c) As is the case with complex substituents in the $—NH_2$ terminus ((d) above), substitution of a complex structure in the —COOH terminal of β-alanine also yields a synaptically active compound, carnosine. However, like β-alanine itself, this substance (β-alanylhistidine) is also a blockader of depolarizing synapses, though weaker in its action. As noted above, however, substitution on the amino side of β-alanine yields pantothenic acid which has the reverse synaptic action. Anserine, a monomethylated carnosine, is synaptically inert.

Dicarboxylic amino acids

Three of these (aspartic, glutamic and α-aminopimelic) were tested. They produced profound depression of all evoked responses as well as reduction in amplitude of 'spontaneous' electrocortical activity (Fig. 14) which appears to have the characteristics of spreading depression. Effects developed slowly and were not immediately reversible unlike those produced by the synaptically active amino acids described above. Depressant effects were similar in many respects to those observed following topical application of KCl. Congeners of these amino acids were, however, synaptically inert (Table 1) except asparagine and γ-guanidinoglutamic, whose actions were similar to those of the dicarboxylic amino acids.

Miscellaneous substitutions

With the exception of cysteine, which had a mild blocking action on excitatory (depolarizing) dendritic synapses, other sulphur-containing amino acids (methionine, taurine) produced no synaptic effects. Similarly the few aromatic and heterocyclic amino acids tested were without significant action (Table 1).

Other Routes of Administration

Effects of intraventricular administration. On the basis of the foregoing data, some of the amino acids were selected for intraventricular injection. With minor exceptions the electrocortical effects produced in this way were similar to those caused by topical applications (Fig. 15). While glycine was relatively ineffective, β-alanine and GABA induced generalized high voltage, slow activity in both hemispheres. The unanaesthetized animals showed characteristics of deep sleep, being aroused by nociceptive or auditory stimulation only with difficulty. On the other hand, δ-aminovaleric acid upon prolonged application induced intermittent paroxysmal activity. Both ε-aminocaproic and ω-aminocaprylic acids produced generalized seizures 10 min after administration. Lysine and cysteine also induced seizures, but after longer periods of action.

Effects of intracarotid injections. This method of delivering GABA to the brain has been reported (MARRAZZI *et al.*, 1958) to produce considerable, though transient depression in the surface-negative component of the transcallosally evoked cortical

potential. Injection of dilantin (diphenylhydantoin) a few minutes before administering GABA was also reported to augment the effectiveness of this synaptic drug.

A series of experiments with intracarotid injections were also carried out in the

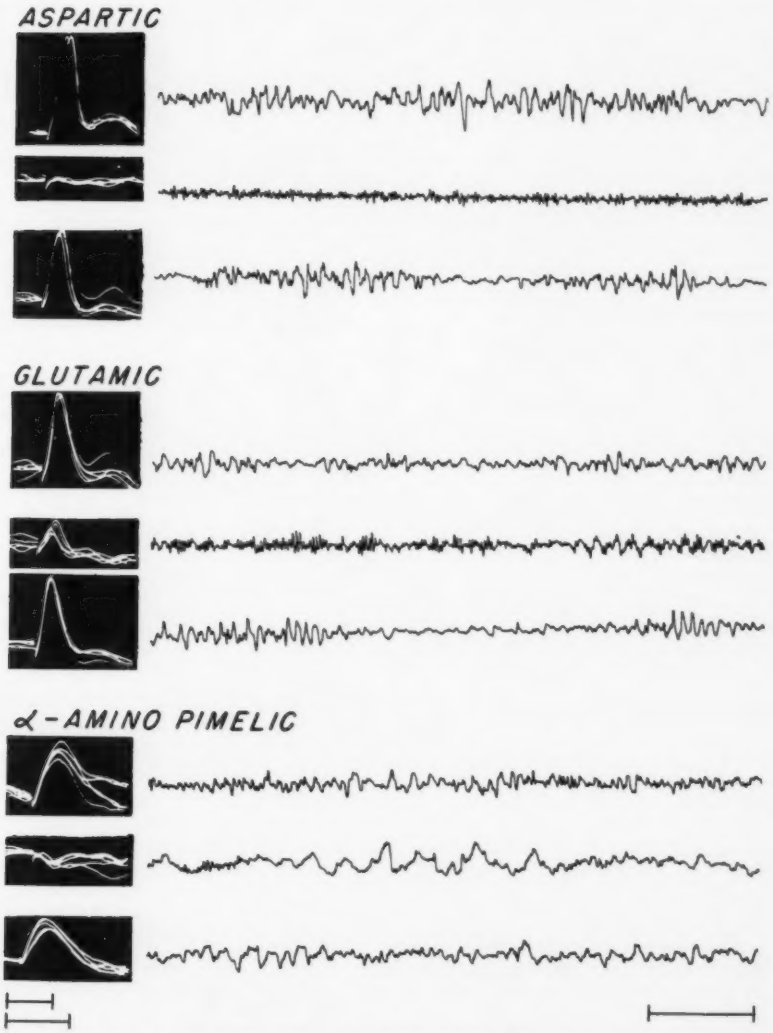

FIG. 14.—Effects of three dicarboxylic amino acids on evoked dendritic responses of cerebral cortex and on simultaneously recorded electrocorticograms. Each series shows the cortical activity before applying the compound (upper line); 5 min after topical application (middle line); and about 30 min after flushing the cortex with Ringer's solution. Time scales: on left, 20 msec, lower of the scales is for α-aminopimelic acid which was taken from a separate experiment. On right, 1 sec for all electrocorticograms.

course of the present work. Not only GABA, but also the oppositely acting ω-amino acids, C_6 and C_8 were tested, both on the transcallosally evoked cortical response and on the directly evoked dendritic potential. The preparations were either succinylcholine-paralysed, or anaesthetized with pentobarbital. A typical experiment is

shown in Fig. 16. Upon injection of 5 mg/kg GABA, the dendritic response to surface stimulation diminished very briefly (for about 8–10 sec). The transcallosally evoked activity was affected even less. Unlike the effects of topical applications, or of systemic injection at regions of blood–brain barrier lesion (PURPURA *et al.*, 1958*b*), intracarotid

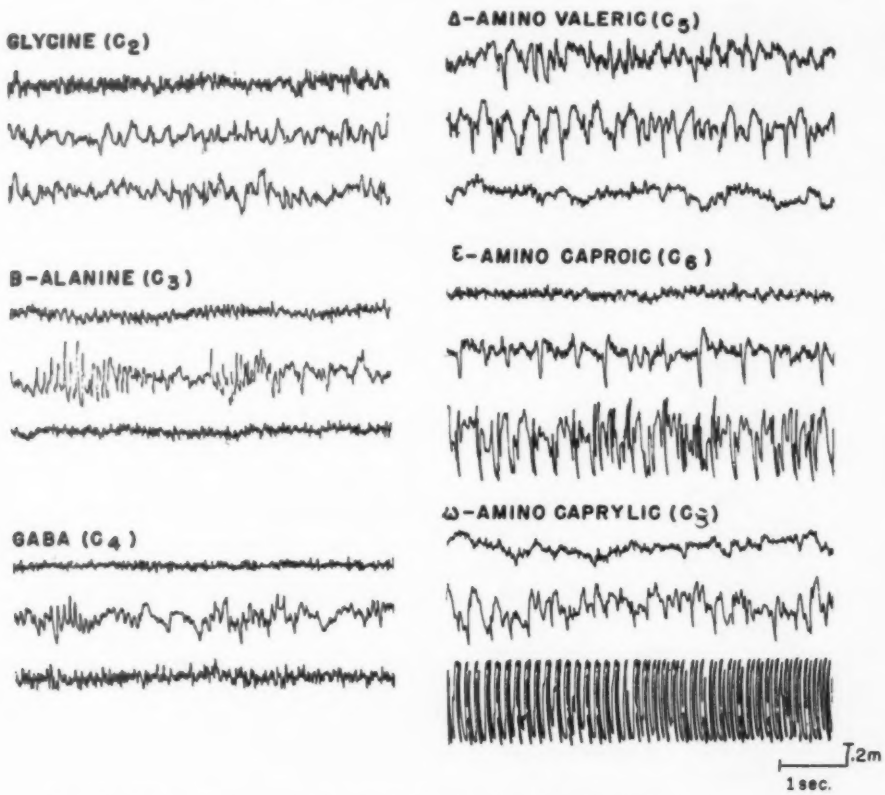

FIG. 15.—Effects of intraventricularly injected ω-amino acids on electrocorticograms. In each series records were made before (upper trace), and after (middle trace) injecting the drug into the ventricles. The amounts were 10 mg in 1 ml for all compounds except C_8, of which only 5 mg were injected. C_2–C_5, lower trace, shows electrocorticograms after flushing ventricles with Ringer's solution. C_6 and C_8 (lower traces) show the maximal development of convulsive activity when drugs were allowed to remain in ventricles for 10–15 min. C_2–C_4 taken from one experiment. The intervals of the recordings are as follows: C_2, initial recording, 15 min after injection and 30 min later (total elapsed time 45 min). After 30 min wait, C_3 was injected, middle record of sequence, at 5 min, lower at 50 min (elapsed time since beginning now 130 min) Then C_4 was administered, middle record of sequence taken 5 min later, and last record at 1 hr. Total elapsed time in this series about 3 hr 15 min, C_5, C_6 and C_8 individual experiments. Middle records taken 30 min, 5 min and 5 min after the injection of the drug in each case. Lower trace for C_5 taken 2 hr after flushing the ventricle.

injection of ε-aminocaproic acid (C_6) or of ω-aminocaprylic acid (C_8) also diminished the evoked surface-negative potentials, but their action was much less than that of GABA. In none of the three experiments, in which this was tested, was an adjuvant action by dilantin observed, even after relatively large amounts (25 mg) of the latter were given. Administration of dilantin in high doses to ambulant animals for 5 weeks prior to the tests also had no adjuvant effect.

DISCUSSION

Characterization of the Mode of Action of the Amino Acids and Related Compounds

Application of differential tests. The starting point of the present analysis has already been pointed out in the Introduction. On the basis of other evidence (PURPURA and GRUNDFEST, 1956*a*, 1957*a*) and of general theoretical considerations (GRUNDFEST, 1957*a*,*b*) it has been concluded that the electrocortical activity recorded from surface leads is primarily or almost exclusively that generated at synapses and

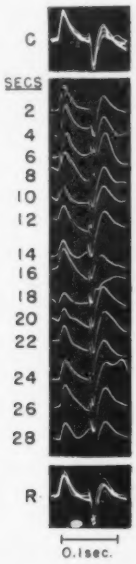

FIG. 16.—Effects of intracarotid injection of GABA on cortical responses to surface and transcallosal stimuli. Pentobarbital-anaesthetized cat (30 mg/kg i.v.). Continuous sequence of records, the surface evoked response appearing first. (C) Five consecutive responses immedately prior to injecting 5 mg GABA in 0·5 ml, followed by 0·5 ml saline solution. The individual traces are shown for responses every 2 sec after the injection. A transient decline, chiefly in the surface evoked response, may have occurred during the eighth to the twentieth seconds. (R) Five superimposed consecutive responses beginning at 30 sec after the injection.

has the nature of postsynaptic potentials of either depolarizing or hyperpolarizing types. The data of this paper do not provide further direct evidence. However, by showing that the various different actions of the drugs may be accounted for in terms of the earlier formulation they enlarge the range of internal consistency of that formulation and consequently provide strong supporting evidence for it. The proof of that consistency lies in the successful accounting, by interlocking differential tests, of the actions of the several varieties of synaptic drugs. These tests are three in number.

(a) The same substance acts differently upon cerebral and cerebellar cortex. This is accounted for on the basis of the conclusion derived from entirely different data (PURPURA and GRUNDFEST, 1957*a*) that the superficial dendritic response of the cerebellar cortex differs from that of the cerebral dendrites by virtue of a relative paucity of hyperpolarizing synapses.

(b) Different substances cause either of two types of effects in the cerebral cortex. Some reverse the evoked dendritic potential, others augment it. This is accounted for on the basis that the two groups of substances are selective inactivators of depolarizing and hyperpolarizing synapses respectively. The evidence for the latter conclusion, likewise, derives from earlier, independently obtained data (PURPURA and GRUNDFEST, 1956*a*, 1957*a*).

(c) The same two groups of substances also produce different effects on the

cerebellar cortex, which are distinct from their actions on the cerebral synapses. The drugs which selectively blockade depolarizing synapses eliminate the dendritic response of the cerebellar cortex, while the second group, the selective blockaders of hyperpolarizing synapses, are strikingly inert upon the cerebellar cortex. These differences are also accountable on the basis of the different synaptic organizations of the cerebellar and cerebral cortex.

The present data show, furthermore, that the two groups of differently acting synaptic drugs may be characterized according to a structural pattern. This suggests that the substances produce these effects by acting upon chemical structures which are linked with the electrogenic properties of different varieties of synapses. The demonstration of sharp differences in the effectiveness of the amino acid drugs for axo-dendritic and axo-somatic synapses is the most clear cut demonstration of an anatomical distinction long stressed by CAJAL (1954).

Mixed actions. It has been stressed elsewhere (PURPURA and GRUNDFEST, 1957*a*) that selectivity of drug action is relative. Strychnine blockade of depolarizing dendritic activity generated by antidromic cortical stimulation clearly illustrates the mixed synapse blocking effects of the convulsant in a pathway devoid of inhibitory substrate. At high concentrations (1 : 500) topical strychnine also blocks surface depolarizing p.s.p.'s of cerebral and cerebellar cortex, a phenomenon paradoxically associated with the rapid development of paroxysmal activity only in cerebral cortex (PURPURA and GRUNDFEST, 1957*b*).* Therefore, the ω-amino acids seem to have very little general blockading action. A concentration of 5% GABA applied continuously to the cerebral cortex for 1 minute still causes reversal of the surface dendritic potential. Elimination of the surface positivity occurs occasionally only with concentrations so high that non-synaptic salt effects may be implicated.

On the other hand, the guanidino acids appear to have markedly mixed actions, since the substances which blockade hyperpolarizing synapses and thereby augment the response in the cerebral cortex also depress the cerebellar surface negativity. A similar action has been previously described in the case of DTC injected into heparinized cats (PURPURA and GRUNDFEST, 1957*a*).

It is to be noted that a mixed action is clearly detectable only in the case of selective blockade of hyperpolarizing synapses, in which the base line of the response in the untreated cerebellar cortex is available. With inactivators of depolarizing synapses there is at present no basis of comparison, since their action is to eliminate cerebellar activity. When the C_3–C_5 ω-amino acids are washed away, there is seen uniformly a transient augmentation of the cerebral dendritic negativity (Fig. 2). The effect suggests that the drugs may also block somewhat the hyperpolarizing synapses, that action reversing less easily than the primary effect on depolarizing synapses. However, other explanations are also possible, based on different kinetics of reversal of the drug action in one or another synaptic pathway.

The kinetics of the action of the drugs upon one or another variety of synapse, and of the reversal of their effects by dilution have not been studied in the present work.

* A striking illustration is the action of strychnine on new born rats in which presumably inhibitory synaptic actions are as yet underdeveloped (cf. MALCOLM, 1955). These animals are highly resistant to intraperitoneal injection of strychnine. They do not develop convulsions at doses lethal for adult rats. With doses up to 100 times the LD_{100} for adult animals, after brief tonic seizures they die in flaccid paralysis if artificially ventilated (unpublished observations), presumably because the curarizing action of the drug in these concentrations blocks all synaptic activity.

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Presumably, such data should give additional specifications for correlating structure with drug action. That the kinetics are different for different drugs and for the same drug with respect to different synapses is indicated by a number of the experiments in the present work. Also not evaluated in the present work are effects which are probably related to kinetics, the interactions of different drugs of this series (PURPURA, GIRADO and GRUNDFEST, 1957*a*).

General depressant effects. The three dicarboxylic amino acids tested (Table 1) all diminished and eventually eliminated cortical responses. Recovery was exceedingly slow. These effects are strikingly in contrast to the selective actions of the ω-amino acids, which develop rapidly and are rapidly reversible. Whether the actions of the dicarboxylic compounds were upon synapses cannot be stated, since the mechanism of spreading depression is as yet unclear.

It is pertinent at this time to stress the distinction between specific synaptic action and non-specific effects which may or may not be operating at synapses. For example, anoxia eliminates synaptic activity long before it blocks conduction in axons. The central effects of systemically administered procaine in mature animals are often marked by convulsions which may develop as a consequence of a selective blockade of inhibitory synapses. Only depressant effects are demonstrable in immature animals (TSOBKALLO and KUCHERENKO, 1954), presumably because of the lack of well developed inhibitory synaptic activity in the latter (cf. footnote, p. 229). On the other hand, elimination of synaptic activity may come about through elimination of electrically excitable, conductile, presynaptic processes. Procaine, though it is somewhat more effective as an inactivator of synapses (GRUNDFEST, 1957*a*,*c*), in its action as a local anaesthetic also effectively blocks conduction.

Topical application of KCl to the cortex probably acts in this last manner. The depolarization of nerve fibres caused by excess K^+ leads to inactivation of electrical responses. Thereby, abolition of excitation to the postsynaptic structures results in abolition of electrocortical activity, although the synaptic membrane is still capable of responding if appropriate means of stimulation are available (GRUNDFEST, 1957*a*,*b*,*c*). Thus, KCl should not be considered a pharmacological agent of synaptic action.

Blurring of these distinctions, as was done by IWAMA and JASPER (1957) led to the conclusion that GABA is a depressant drug, but with its action restricted to the superficial cortical layers. The authors state, however, that the simplest explanation of their results would be the interpretation presented here and in earlier publications (PURPURA *et al.*, 1957*a*,*b*, 1958*a*; PURPURA and GRUNDFEST, 1956*a*, 1957*b*), that GABA 'may be a specific synaptic blocking agent' (IWAMA and JASPER, 1957, p. 378).

Sites of action of the drugs

The data presented in this paper show that the effects of the amino acid drugs are exerted chiefly and perhaps exclusively on the axo-dendritic synapses. This indicates a new form of specificity of chemical structures in different varieties of synapses. Ample pharmacological data are available that some agents act more effectively upon one function of the central nervous system or another (e.g. antipyretics, analgesics, etc). In the present case, however, the distinction is clearly between two different membrane structures of the same cells. Thus, the dendritic negativity evoked by antidromic invasion of pyramidal neurones is eliminated by GABA. However, the axo-somatic

synaptic response of the cells, which leads to their repetitive discharge into the pyramidal tracts is not affected.

In view of the numerous findings regarding specific actions of drugs (e.g. on excitatory or inhibitory; adrenoceptive or cholinoceptive; muscarinic or nicotinic synapses) it is not surprising that another pharmacological differentiation between axo-somatic and axo-dendritic synapses is also found. The differential sensitivity of axo-dendritic as opposed to axo-somatic synapses to the psychotomimetic agent LSD-25 has been previously described (PURPURA, 1956, 1957*a*). Still other pharmacological differences are to be expected and have been noted (SIGG and GRUNDFEST, 1958). These should provide a basis for a comparative pharmacology of synapses.

The effects of several of the compounds listed in Table 1 have been analysed with intracellular recordings and conductance measurements in lobster muscle fibres (GRUNDFEST, *et al.*, 1958, to be published). The excitatory and inhibitory p.s.p.'s of the neuromuscular synapses were evoked separately by stimulating individually the respective axons. GABA and all related short-chain compounds that were tested increased the membrane conductance by activating the inhibitory synapses. The longer-chain compounds were inert, but carnitine, which shares their effects on cat cortex, activated the excitatory synapses. Picrotoxin blocked the inhibitory synapses specifically. In the lobster and probably also in crayfish muscle fibres (VAN DER KLOOT *et al.*, 1958) and stretch receptors (ELLIOTT and FLOREY, 1956; EDWARDS and KUFFLER, 1957) GABA and picrotoxin are competitive antagonists as they are in cat brain (Fig. 4), but by different mechanisms. In the vertebrate, picrotoxin activates the excitatory synapses which GABA inactivates. In the crustaceans, picrotoxin is the inactivator and GABA the activator, but of inhibitory synapses. This doubly inverted relation is restricted, however, since acetylcholine, DTC, strychnine and other vertebrate pharmacological agents are without effect on the lobster neuromuscular synapses.

Another element in this comparative scale may be mentioned here. Several varieties of vertebrate sensory receptors that have been tested, bullfrog olfactory epithelum and retina (SIGG and GRUNDFEST, 1958), appear to be inert to the amino acids, whereas the dendrites of the crayfish stretch receptor respond to the drugs which blockade depolarizing electrogenesis, but not to those that blockade hyperpolarizing dendritic synapses (BAZEMORE *et al.*, 1956; EDWARDS and KUFFLER, 1957; ELLIOT and FLOREY, 1956). A similar relation also holds for neuromuscular synapses. Activity of vertebrate skeletal muscle is not affected by massive injections of GABA or similarly acting drugs. On the other hand, neuromuscular transmission in crayfish is blocked by GABA (MCLENNAN, 1957). Upon intravenous injection of GABA and other compounds in mammals, marked changes have been noted in the blood pressure (IINO, 1955; TAKAYASU, 1956) and on cardiac rhythmicity (JASPER, 1957). This suggests that these drugs may act on vertebrate vascular smooth muscle and, perhaps, on cardiac synapses.

The predominantly axo-dendritic site of action of the amino acids demonstrated in the present work also accords with the finding of MCLENNAN (1957) that when GABA is applied to the spinal cord of cat it does not affect the knee jerk. The same observer, however, reported that GABA protects mice against strychnine convulsions (MCLENNAN, 1957), whereas in our experience (PURPURA, GIRADO and GRUNDFEST, 1957*b*) there is no protection. Strychnine acts not only upon the dendrites, but also on the axo-somatic synapses (cf. ECCLES, 1957; KUNO, 1957). While convulsive

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motor activity may be produced by the more powerfully acting amino acid drugs, it has also been shown in the present work that GABA and its congeners do not block the axo-somatic excitatory synapses, the means through which convulsive strychnine action can be lifted by blockaders of depolarizing synapses. Our finding (in two series of experiments with twenty-five mice in each series) that GABA in no case affects the threshold for strychnine convulsion, nor in any other manner protects mice against strychnine, thus accords with other types of data. It may be noted in this connexion that while some samples of factor I were reported to protect mice against strychnine convulsions (FLOREY and MCLENNAN, 1955) other samples had no such action (ELLIOTT and FLOREY, 1956). A sample of crude but potent factor I (kindly provided by Dr. A. BAZEMORE) was tested in the course of the present work and also proved ineffective for protecting mice against strychnine.

Access of amino acids to the central nervous system

Another reason why intraperitoneal injection of GABA or its congeners would not be expected to protect against strychnine is the restricted access of the amino acids to the central nervous system. Systemic applications of strychnine reach the synapses of the neuraxis, but similarly administered amino acids do not. These findings regarding the barrier have been confirmed by IWAMA and JASPER (1957) and partially also by MARRAZZI *et al.* (1958), since the effects of intracarotid injections of GABA which the latter described were transient. As was noted above, in our own experiments intracarotid injections resulted in very minor depressions of cortical activity, not only by GABA, but also by injections of the agents which augment the surface-negative potentials on topical application (C_6, C_8).

The reality of the blood–brain barrier to the amino acids is established by the dramatic effects produced when these drugs can gain access to the cortical synapses through the circulation in local regions of blood–brain barrier lesions (Fig. 6; cf. PURPURA *et al.*, 1958*a*,*b*). The use of such localized sites of access has also permitted confirmation that the amino acids act primarily and, perhaps, exclusively on the axo-dendritic synapses (Fig. 10).

Comparison experiments in barrier-intact and barrier-damaged cortex afford a new, functional test for the penetration of drugs into the central nervous system. Besides providing, in the effects on electrical responses, a new criterion of penetration, the rapidity of electrical measurements can cope with the apparently high rate of penetration of the drugs.

Role of amino acids and related synaptic drugs

It was already noted that a number of the compounds whose synaptic actions have been described in this paper occur normally in the body and that some are particularly related to the central nervous system. The fact that these compounds are best tested by their topical application to the brain is an incidental matter, forced by their failure to penetrate effectively the series of membranes constituting the so-called blood–brain barrier. This should not, however, detract from their candidacy as possible transmitter agents for synaptic activity of the central nervous system.

Release of transmitter chemicals from presynaptic nerve terminals is presumably the general mode for mediating synaptic activity. Substances like GABA, β-alanine, or γ-guanidinobutyric acid (PISANO and UDENFRIEND, 1958) which occur in and are

synthesized by the brain may be available in this type of close range action, since release at presynaptic terminals would bypass the blood–brain barrier to these synaptic drugs. In that topographic relation the blood–brain barrier would, indeed, serve to prevent these relatively stable compounds from exerting widespread circulatory actions.

Thus, those synaptic drugs that are normal constituents of the brain, may play a role in synaptic activity. If they are produced by the influence of presynaptic nerve impulses in close relation to the synaptic membrane, they would have to be designated as transmitters. However, the type of transmitter action they would exert would be different from that of substances like acetylcholine or the epinephrine compounds. The latter are synapse activators (GRUNDFEST, 1957*a*), excitants and initiators of synaptic electrogenesis. The amino acid 'transmitters,' if such they are, all appear to be inactivators of synapses. The possibility that the central nervous system also has transmitters which inactivate synapses would extend the range of control of synaptic interaction. An inactivator of depolarizing, excitatory synapses would have an 'inhibitory' action, but this would be exerted by counteracting membrane activity at its origin, by preventing or diminishing the depolarizing transducer action, rather than by activating an oppositely electrogenic transducer, as is the case when inhibition of depolarizing synapses is obtained by exciting the hyperpolarizing synapses. The blockaders of hyperpolarizing synapses on the other hand, would exert overt 'excitatory' effects, as does strychnine, by diminishing the inhibitory electrogenesis of the hyperpolarizing synapses.

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Synaptic action of vitamins

Several of the B-group vitamins are listed among the synaptic agents in Table 1. These findings raise a question regarding the range of effects of the vitamins. For example, thiamine is implicated as a coenzyme in various aspects of intermediary metabolism and thiamine deficiency produces many other symptoms of malfunction, as well as the neurologic signs. However, in cats which are moribund in extreme thiamine deficiency, recovery upon injection of thiamine is a matter of only a few minutes,* probably long before the metabolic deficits can be made up. The synaptic effects of thiamine could account for this rapid action, which is particularly centred upon the neurological symptoms.

The chemical pathways and kinetics of surface pharmacological actions may differ radically from those of metabolism. Recognition of a pharmacological synaptic role of vitamins therefore would seem to be an important matter (GRUNDFEST and PURPURA, 1957; PURPURA, GIRADO and GRUNDFEST, 1958*c*). This also applies to the roles of other naturally occurring substances, of which creatine, creatinine and arginine are particularly to be noted.

Chemical structure and pharmacological actions

The present work adopts a common device, laying emphasis on the zwitterion structure of ω-amino acids in seeking a convenient means for classifying their relation to function. There is, however, no evidence that this structural relation is, indeed,

* Unpublished experiments during 1942–1943 at the Rockefeller Institute for Medical Research by one of us (H.G.) and Dr. Charles NEUMANN.

the key link and the present use of the device is chiefly descriptive. Nevertheless, a few deductions may be derived tentatively from the available data.

The most striking feature of the several series of synaptic drugs studied in the present work is the restriction of their action to axo-dendritic synapses. This finding indicates that the chemical structures of electrogenic membrane at axo-somatic synapses differ in some characteristic, but as yet unknown manner, from those of axo-dendritic synapses of the same cells.

The selective action of shorter chain compounds primarily on depolarizing synapses and of the larger chains on the hyperpolarizing are subject to a number of modifications. Further analysis of these factors may supply other specifications on the structures of the different electrogenic synaptic membranes.

(a) All the compounds studied thus far have considerable rotational freedom. If the separation between anionic and cationic sites is a key relation, each of the compounds may have had an overlapping range of distances. Studies are underway with substances that have fewer degrees of rotational freedom, such as unsaturated ω-amino acids.

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(b) Not only the distance between the zwitterion poles may be important, but also the electrostatic charge distribution at each pole probably plays a part. Thus, a greater degree of modification in structure appears to be permissible in the guanidino acids than in the corresponding amino acids. A striking example is the finding that the α-guanidino compounds retain synaptic action, while α-amino acids are without effect. Similarly, introduction of hydrocarbon branches, while altering synaptic action in the case of guanidino compounds, abolished activity of the ω-amino acids.

(c) Another type of example is to be found in the synaptic action of β-alanine and two of its derivatives, carnosine (β-alanyl-L-histidine) and pantothenic acid (pantoyl-β-alanine). Carnosine, like β-alanine, but less strongly, blocks depolarizing synapses. The complex substituent added at the other terminus of β-alanine converts the synaptic action to blockade of hyperpolarizing synapses. It is surprising that anserine is synaptically inert since the methyl group which distinguishes this dipeptide from carnosine is attached to the imidazole ring.

(d) Several other examples of alteration of the type of synaptic effect are also listed in Table 1. All of these also represent compounds in which the positively charged terminal group has been modified; γ-aminobutyrobetaine; carnitine (γ-amino β-OH butyrobetaine); creatine; and creatinine.

(e) It is perhaps significant that we have found no reversal from a blockader of hyperpolarizing to one acting on depolarizing synapses.

The observations may be generalized tentatively as follows: Short chain length between the zwitterion poles predisposes to selective action on depolarizing synapses. Increased polarization of the terminal groups achieved either by their greater separation or by substituting more strongly electrically charged terminal groups predisposes to selective action on hyperpolarizing synapses. The synaptic actions of the ω-amino acids can be codified within the framework of this formulation. The low potency of glycine probably indicates both that the short chain length is inappropriate for combining with the receptor sites of the synaptic membrane and that interaction between the close, oppositely charged termini tends to neutralize both. The α-amino acids, all of which may be considered as alkyl-substituted glycines lose even the little potency of glycine by virtue of the further weakening of the positively charged site.

The next two compounds, β-alanine (C_3) and GABA (C_4) are about equally effective, while the C_5 compound appears to be less active. This may be due to stronger divalent action of the C_5 compound which is indicated by the fact that δ-aminovaleric acid on intraventricular administration initiates convulsant activity (Fig. 15). Simultaneous and approximately equal blockade of both types of synapses in the cerebral cortex may result in decreased responses without inversion of the potential This could not be tested in the present work since a drug of this action (e.g. DTC) also blocks cerebellar cortical activity like the selective blockaders C_3 and C_4 do. Another possibility, that the chain length of the C_5 compound is inappropriate to the structures of either type of synapse therefore cannot be evaluated.

The hyperpolarizing synapses are attacked by compounds with a much broader spectrum of structures. Their carbon chain length ranges from C_6 (ε-aminocaproic acid; γ-guanidinobutyric acid) to the largest studied, a C_{11} equivalent (ω-guanidinononanoic acid). It may be noted at this point that radical changes of function have been noted in various series of chemotherapeutic agents as the carbon chain length equals or exceeds C_5 (BARLOW, 1955). In the present case, however, the rotational freedoms of the molecules studied preclude decision whether the greater length is itself the important determinant of greater synaptic potency. However, that this is not the situation is suggested by several cases among the inversions of action mentioned earlier. The betaine structure, of γ-aminobutyrobetaine or of carnitine, is not likely to lengthen the effective chain length of GABA itself. Likewise, the methyl substitutions in guanidinoacetic acid to produce creatine and creatinine probably do not expand the chain length of the parent substance. In both cases there is probably an increase in the charge of the positive terminus, and this then must be considered a prime factor in the synaptic action.

The above formulation also accounts satisfactorily for the effects of ω-guanidino acids. The positively charged terminus is probably more strongly so than the amino end of the corresponding ω-amino acids. It is probably this factor which accounts for the persistence of synaptic action in the substituted guanidinoacetic compounds (creatine, creatinine, α-guanidino acids). Presumably the loss of polarization at one end by adding an α-amino group (arginine) or at the other by changing γ-guanidinobutyric acid to ureidobutyric acid (Table 1) is the factor which leads to loss of synaptic action.

Cyclization of GABA into its lactam also destroyed synaptic action, but in creatinine and ε-caprolactam the actions of the straight chain homologues are retained. This suggests that closer study of such compounds may reveal critical data which are not available at the present time. An interesting fact which also may have further implications is the ability of systemically administered ε-caprolactam to produce synaptic action. This indicates that the substance penetrates the blood–brain barrier.

SUMMARY

(1) The actions of a number of amino acids and related compounds as synaptic drugs have been characterized by differential tests emphasizing their effects upon cerebral and cerebellar electrocortical activity. An explanation of their effects in terms of chemical structure is tentatively proposed.

(2) The ω-amino acids of carbon length two to five inclusive are selective inactivators of depolarizing, excitatory synapses of superficial dendrites. The

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longer chain ω-amino acids inactivate the hyperpolarizing, inhibitory axo-dendritic synapses.

(3) The ω-guanidino acids and to a lesser extent the α-guanidino acids, but not the α-amino acids, are similarly active. The ω-guanidino compounds behave synaptically as if the true number of carbons in the $-CH_2-$ chain were increased by 2. This appears to be due to additions of the —NH—C— link

(4) Addition of an α-amino group to the ω-amino or ω-guanidino acids weakens their potency as synaptic drugs.

(5) Other compounds with synaptic action may be related structurally to the factors presumed to be responsible for the synaptic actions of the ω-amino and ω-guanidino acids.

(6) The types of synaptic action of these substances appear to be linked with their zwitterion structure. Compounds of chain lengths longer than five carbons or possessing termini more strongly polarized than have the C_2 to C_5 ω-amino acids are the inactivators of hyperpolarizing synapses.

(7) All but one of the compounds tested were ineffective by systemic administration (intravenous or intracarotid injections) indicating their inability to penetrate the blood–brain barrier. In regions of local barrier lesions the effects produced by systemically injected drugs were the same as those caused by topical applications.

(8) Circumventing the blood–brain barrier by intraventricular injections of the ω-amino acids resulted in synaptic effects.

(9) The drugs act selectively upon axo-dendritic synapses and affect the axo-somatic little or not at all.

(10) Their possible role as synaptic 'transmitters' of a new type is discussed. Of particular relevance in this connexion is the occurrence and production of some of these agents (e.g. γ-aminobutyric acid and γ-guanidinobutyric acid) in the brain.

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A MAJOR COMPONENT OF NEUROHYPOPHYSIAL TISSUE ASSOCIATED WITH ANTIDIURETIC ACTIVITY

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THE hormonal activity of the hypothalamico–hypophysial system has been correlated with substances of three widely differing orders of complexity: the peptide hormones (DU VIGNEAUD, GISH and KATSOYANNIS, 1954), the van Dyke protein (VAN DYKE, CHOW, GREEP and ROTHEN, 1942) and the neurosecretory material (SCHARRER and SCHARRER, 1954). LANDGREBE, KETTERER and WARING (1955) have calculated that a complex consisting of one molecule of each peptide bound to one molecule of the van Dyke protein would have biological activity equivalent to that found by van Dyke and his colleagues. According to these calculations, the secretory product should comprise 12 per cent of the dry weight of the gland. Conversely, the individual peptide hormones could each constitute no more than 0·4 per cent of the dry weight. From histological studies, SMITH (1951) estimated 25–35 per cent of the total bulk of the neurohypophysis to be neurosecretory material. However, the evidence for the protein nature of the secretory product has been indirect. Thus, after a study of the mode of association between the peptide hormones and the van Dyke protein, ACHER, MANOUSSOS and OLIVRY (1955) conclude that because of the preparative procedures "it is impossible to define the mode of association in order to decide whether it is an artifact or, on the contrary, an association of profound biological significance."

The following experiments present additional evidence that the biologically active material which is secreted from the neurohypophysis in response to restricted water intake is secreted in association with a protein-like material which in well-hydrated rats consitutes a major fraction of the total protein-like material of the gland.

MATERIALS AND METHODS

Posterior pituitary material was obtained from male Osborne–Mendel rats weighing from 145 to 180 g initially. The control series received water *ad lib*. The hydrated rats received 3% glucose as drinking water for 7 days prior to sacrifice. The dehydrated animals were maintained on a restricted water intake (approximately 8 ml per day) for 4 weeks prior to sacrifice. The weight loss of the dehydrated rats was from 13 to 28 g. At appropriate times the animals were decapitated and the hypophyses and anterior hypothalami were removed and placed in liquid nitrogen within from 4 to 6 min after decapitation.

Frozen-dried 30 μ sections of the neurohypophyses and hypothalami were prepared according to the procedure of LOWRY (1953). Samples were dissected from the sections under a low power stereomicroscope and weighed on a quartz fibre microbalance.

The samples, weighing from 5 to 10 μg, were placed in test tubes of 3 mm bore and extracted three times at room temperature with 50 μl of absolute alcohol followed by 3 $\times$ 50 μl of hexane to remove lipids and low molecular weight material. When subsequent extractions were made with an aqueous solvent, the samples were afterwards dehydrated with absolute alcohol and hexane. The order of the

successive fractionations was always from higher to lower ethanol concentrations. Fractions of material for the bio-assays were taken to dryness in a centrifuge evaporator (ALBERS and LOWRY, 1955) and stored at −20°c. The solvents designated as *x* per cent ethanol–1 per cent acetic acid were prepared from *x* ml absolute ethanol plus 1·0 ml glacial acetic acid and diluted to 100 ml final volume with glass distilled water.

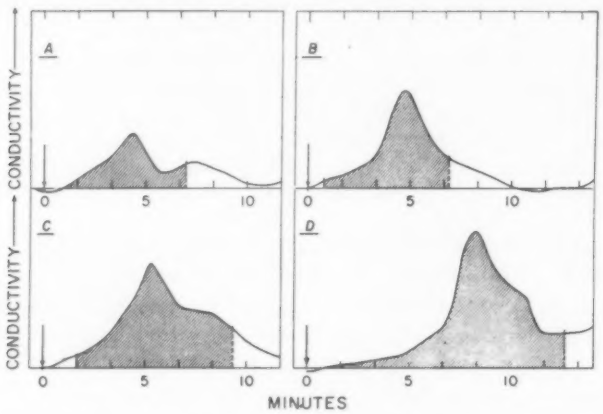

FIG. 1.—(A) and (B) are records of the urine conductivity responses to 75 and 150 micro-units of pitressin. (C) and (D) are the records of the urine conductivity responses to aliquots of the 75–85 per cent neurohypophysial tissue fraction of a rat which had received water *ad lib*. (C) is the response to an aliquot equivalent to 33 mμg dry weight of tissue; (D) is the response to an aliquot equivalent to 66 mμg of tissue. The shaded area under the curves is the the portion between the tenth and fiftieth post-injection drop of urine.

Relative values for dry mass per unit volume of tissue were calculated from the weight of the sample and the area of *camera lucida* tracings of its outline. Since the absolute thickness of sectioned material is difficult to ascertain, only relative density values were calculated.

The bio-assay for antidiuretic activity is a modification of the intravenous rat method of DICKER (1953). Female Osborne–Mendel rats weighing 180–230 g were used as assay animals. The animals were initially anaesthetized with ethanol and hydrated as described by DICKER. Polyethylene catheters were inserted in the urethra, the femoral vein, the rectum and the peritoneal cavity. The rat was kept under light anaesthesia during the assay by intraperitoneal 25 per cent ethanol. A constant state of hydration (about 10 per cent of body weight) was maintained with 1:30 dilute Ringer solution administered rectally. Photoelectric drop counters regulated the infusion rate from a motor-driven syringe and recorded the rate of urine flow.

CRAWFORD and PINKHAM (1954) employed freezing point depression in an antidiuretic assay and discussed certain theoretical advantages of their method over rate measurements. For a continuously recording assay such as the Dicker method such measurements are not practicable. However, we have found that urine conductivity is a convenient measure of electrolyte concentration. Electrolyte concentration under constant water load is found to be an inverse function of the rate of urine secretion.

A constant current 60 c source of about 1 ma. is applied across two stainless steel inserts in the polyethylene urethral catheter. The voltage drop across a 25 mm segment of polyethylene tubing between the inserts is rectified and recorded by a Sanborn recorder. Antidiuresis is reflected in an increased urine conductivity and a decreased voltage drop (Fig. 1). The major part of the conductivity response was found to occur between the tenth and fiftieth drop of urine recorded after the time of sample injection. The area under this part of the conductivity curve was taken as a quantitative measure of the response.

The antidiuretic activities of the neurohypophysial fractions were estimated by a four point assay similar to that described by HOLTON (1948) for oxytocin (Fig. 2). Standard solutions were prepared from Pitressin (Parke–Davis) obtained through the courtesy of Dr. D. A. MCGINTY.

RESULTS

In a preliminary experiment, four samples of neurohypophysis taken from sections of a single pituitary gland were extracted with alcohol plus hexane and then with 50 per cent alcohol containing 1 per cent acetic acid. The result shown in Table 1 demonstrates that a large fraction of the non-lipid material, presumably proteins and

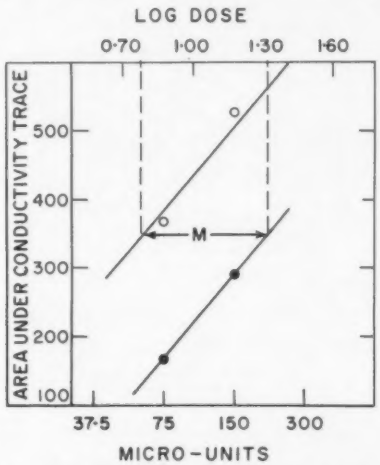

FIG. 2.—The areas of the records of Fig. 1 are plotted as described by HOLTON (1948). The black circles are the standards. The responses to the unknown samples (open circles) are plotted on the same abscissas as the standards. The distance, *M*, is then taken as the logarithm of the ratio of potencies. In this case, *M* corresponds to a ratio of 3·5 and the unknown assays (3·5 × 0·150)/0·066 = 7·9 milliunits/μg.

peptides, was soluble in acidified 50 per cent alcohol. This fraction was much larger than that found in either anterior pituitary gland or hypothalamus (Table 2). Less than 1 per cent of additional material was removed by 1 per cent aqueous solution of acetic acid.

TABLE 1.—FOUR SAMPLES OF NEUROHYPOPHYSIS FROM A SINGLE RAT PITUITARY

Sample	Total dry weight (μg)	Material removed by hexane + ethanol		Material removed by acidified 50 per cent ethanol		
		(μg)	(% of total)	(μg)	(% of total)	(% of non-lipid)
1	6·78	1·11	16·3	2·33	34·3	41·1
2	9·22	1·58	17·2	3·60	39·1	47·2
3	6·35	1·32	20·8	2·33	36·7	46·2
4	6·08	1·49	24·5	2·18	35·9	47·2

A similar fractionation of material from glucose hydrated, control and dehydrated animals was carried out (Table 3). Although the size of this soluble fraction increased only slightly over the control value in hydrated animals, it was markedly reduced in the dehydrated animals. As might be expected, the standard error of the control group was larger than that of the hydrated group. The dry mass per unit volume was about 13 per cent less in the glands from dehydrated animals (Table 4).

The neurohypophysial dry weight is apparently composed of a constant and a variable component when the experimental variable is water intake. It seemed desirable to use a reference weight which does not itself include the variable component. For this reason the weight of the insoluble fraction remaining after the last extraction with 50 per cent ethanol is used as the reference weight in the subsequent experiments.

TABLE 2.—FRACTIONATION OF THREE DIFFERENT RAT TISSUES

	Mean and S.E.M. of five samples	
	Percentage of total dry wt. extracted by ethanol + hexane	Percentage of non-lipid material extracted by acidified 50 per cent ethanol
Lateral hypothalamus	31·1 ± 1·5	16·7 ± 2·1
Anterior pituitary	12·3 ± 0·6	29·4 ± 1·3
Neurohypophysis	23·2 ± 0·9	43·2 ± 0·4

The preceding results from a total of twenty-eight animals appeared to be sufficiently reproducible to warrant a more discriminative fractionation procedure. A 'solubility profile' was obtained by successive extractions with acidified alcoholic solvents of progressively increasing water content. The solubility profiles of neurohypophysial tissue from hydrated rats and those from dehydrated rats differed principally in the amount of material which was insoluble in acidified 85 per cent alcohol but

TABLE 3.—RAT POSTERIOR PITUITARIES: PERCENTAGE OF NON-LIPID MATERIAL SOLUBLE IN 50 PER CENT ETHANOL–1 PER CENT ACETIC ACID

	Mean	S.E.M.	*n*	*P*
Ad lib 3 per cent glucose	47·3	0·9	9	>0·05
Ad lib water	43·8	1·9	9	<0·001
Restricted water	22·6	1·0	8	

soluble in acidified 75 per cent alcohol (Fig. 3). This material will hereafter be referred to as the 75–85 per cent fraction. The fact that this fraction nearly disappeared in the dehydrated animals indicates that essentially all of this fraction is related to the endocrine secretion of the tissue.

TABLE 4.—DRY MASS PER UNIT VOLUME OF NEUROHYPOPHYSIAL TISSUE

	Ad lib water	Restricted water
Relative dry mass per unit volume (arbitrary units)	251	218
S.E.M.	5·8	2·3
n	4	4
P	<0·01	

The pars intermedia and pars anterior of the hypophysis and the lateral preoptic nucleus of the hypothalamus contained much smaller amounts of material in the 75–85 per cent fraction (Fig. 4).

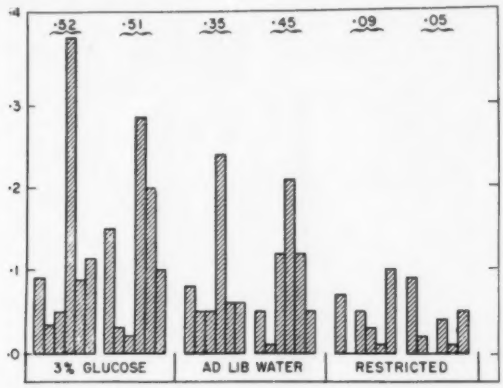

FIG. 3.—Solubility profiles of neurohypophysial tissues. Each group of six bars represents the fractionation of a sample from one rat. The six bars represent, from left to right, the amount of material removed from the defatted tissue by successive extractions with 95 per cent, 90 per cent, 85 per cent, 80 per cent, 75 per cent and 50 per cent ethanol (*v*/*v*). Each solvent contained 1 per cent acetic acid. The ordinate is the ratio of the weight of material removed to the weight of the sample after extraction with acidified 50 per cent ethanol. The figures above the braces are the total ordinate values for the 75–85 per cent fraction.

The individual alcohol fractions of neurohypophysial tissue were assayed for antidiuretic acitvity (Fig. 5). The activity was found to be largely confined to the 75–85 per cent fraction and was markedly reduced in the glands of water-restricted animals.

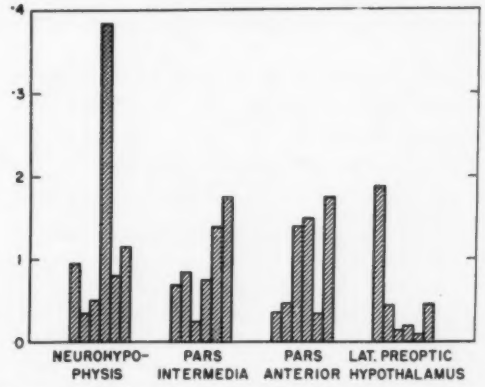

FIG. 4.—Samples were taken from four tissues of the same rat. Ordinate and abscissa are the same as in Fig. 3.

This result was confirmed in a second series of animals (Table 5) in which the amount of 75–85 per cent fraction is compared with the biological activity in control and water-restricted animals.

DISCUSSION

The present evidence for the association of some 20 per cent of the fat-free dry weight of the neurohypophysis of the rat with the secretory product is in agreement with

the calculation of Landgrebe *et al.* (1955) for the content of van Dyke protein in beef posterior lobes. This finding cannot be reconciled with the hypothesis that the peptide hormone is secreted unassociated with other material. The content of peptide hormone is estimated to be 2 mg/g of beef gland acetone powder (WINNICK, WINNICK,

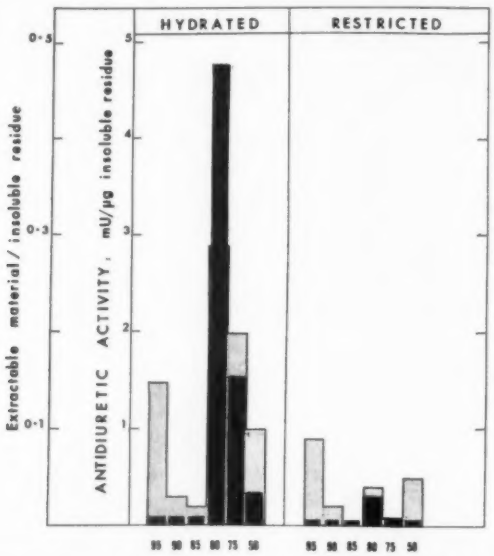

FIG. 5.—Comparison of the distribution of antidiuretic acitvity and the solubility profile in the neurohypophysis of a hydrated rat and a rat subjected to water restriction. The stippled bars represent the relative weights of extractable material, as in the preceding histograms. The superimposed black bars represent the relative biological activity of each fraction. Both ordinates are referred to the weight of the sample after the last extraction.

ACHER and FROMAGEOT, 1955). Neurohypophysial tissue is known to contain a large pool of peptides. However this amounts to only 3·4 per cent of the dry weight of hog glands (RAMACHANDRAN and WINNICK, 1957).

Histochemical studies have characterized the neurosecretory colloid variously as a 'glycolipo-protein complex' (SCHIEBLER, 1952), 'somewhat like a lipoprotein' (HILD and

TABLE 5.—COMPARISON OF ANTIDIURETIC ACTIVITY AND CONTENT OF 75–85 PER CENT FRACTION IN CONTROL AND DEHYDRATED RATS

	Antidiuretic activity (milliunits/µg insoluble fraction)	Content of 75–85 per cent fraction % of insoluble fraction)
Ad lib water		
Mean	10·5	37·3
S.E.M.	2·5	4·05
n	6	6
Restricted water		
Mean	0·31	2·9
S.E.M.	0·05	1·0
n	6	6

ZETLER, 1953), 'protein or polypeptide in character; the presence of lipid or carbohydrate is excluded' (HOWE and PEARSE, 1956), and 'not a glycolipoprotein, but a protein rich in cystine' (SLOPER, 1955). In interpreting such observations one should be aware of the electron microscopic studies which indicate that the large granules which are revealed by light microscopy are an artifact arising from the clumping together of many smaller granules (approximately 0·1 μ diameter). Each of these small granules is composed of a peripheral membrane and a central homogeneous material (PALAY, 1957; BARGMANN, KNOOP and THIEL 1957). It is reasonable to suppose that the membrane is of different chemical composition than the material contained within the membrane. Some of the conflicting histochemical observations may result from the structural heterogeneity of the granules.

HILD and ZETLER (1953) concluded that the neurosecretory material, as demonstrated by the Bargmann technique, is extracted from fresh pig neurophpophyses by an ethanol–chloroform solvent. In view of our data on the solubility of material associated with neurosecretion in the rat, the amount of water contributed to the solvent by fresh tissue may have been an important factor. However, the component of neurosecretory material which is stained by the Bargmann technique may not be identical with that which we have studied. They also report that the biological activity remains in the tissue after their extraction procedure. ACHER *et al.* (1955) have demonstrated that the peptide hormones may be removed from the protein of van Dyke, but the treatments which they find necessary are more severe than a single extraction.

The isolation of the van Dyke protein containing both oxytocic and antidiuretic activities has been regarded as evidence for a unitary mode of neurohypophysial hormone secretion (LANDGREBE *et al.*, 1955), although the bulk of physiological experiments have indicated selective depletion of one or the other activity in response to the appropriate stimuli (DICKER and TYLER, 1953; OLIVECRONA, 1954; ABRAHAMS and PICKFORD, 1954). At present, we have no evidence for the concomitant loss of oxytocic acitivity with the 75–85 per cent fraction; however, this loss would be implied if this fraction were accepted as analogous to the van Dyke protein.

Since the depletion of this fraction is so nearly complete in response to water restriction, the alternatives to the association of oxytocin with this same material are either an association of the oxytocin peptide with a material of considerably different physical properties or the presence of oxytocin in the neurohypophysis as the free peptide. The free peptide would not constitute a sufficiently large proportion of the non-lipid material to be detectable by the fractionation technique which we have employed.

There is some evidence that the peripheral activity of these hormones may be regulated by factors in plasma (HAWKER, 1957). Such factors might render the hypothesis of parallel secretion compatible with selective activation of the target organs.

The results presented here can be taken to establish only that during water deprivation in the rat a greater bulk of material is lost from the neurohypophysis than can be accounted for by assuming secretion of the active peptide hormone alone. The solubility of this material in aqueous alcohol is shared by the van Dyke protein (VAN DYKE *et al.*, 1942), the preparations of ABEL (1930), and the stainable neurosecretory material (Wingstrand). The content of this 75–85 per cent fraction in well-hydrated rats is in quantitative agreement with the content of van Dyke protein in beef glands (WARING and LANDGREBE, 1950) while the marked decrease during water deprivation is similar to that observed for neurosecretory material (SCHARRER and SCHARRER, 1954)

SUMMARY

More than 40 per cent of the non-lipid material of the lyophilized rat neurohypophysis is soluble in 50 per cent ethanol–1 per cent acetic acid. Glands from rats subjected to chronic water restriction contain about half as much material soluble in this solvent. The decrease in this soluble substance is accompanied by a 13 per cent decrease in the dry mass per unit volume of tissue. The material which disappears during water restriction is insoluble in acidified 85 per cent ethanol but soluble in acidified 75 per cent ethanol, and accounts for nearly all of this fraction. The antidiuretic activity of the gland is also confined to the 75–85 per cent fraction and disappears from the gland along with this fraction during water deprivation.

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RELATIVE RESPIRATION OF NEURONAL AND GLIAL CELLS*

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INTRODUCTION

THE suggested functions for glial cells in myelinogenesis (LUSE, 1956) and active transport of nutrients from vasculature to neurones (WINDLE, 1958) have raised the issue of the energetic requirements of these cells. The assumption that nerve cells are responsible for the major portion of oxygen consumption by the brain is not seriously questioned but the extent to which the various structural components—neurones, non-neuronal cells and axoplasmic constituents—contribute to respiration has not been established. In a previous communication (KOREY, ORCHEN and BROTZ, 1958) it was shown that the cells of a neuroglial concentrate from white matter of lamb brains could respire at a rate equivalent to $9{\cdot}2–12{\cdot}5 \times 10^{-6}$ μl/hr per cell. Of the total respiration of homogenates of white matter about one-third could be attributed to the respiratory activity of the axoplasmic structures coursing through the white matter and two-thirds to the cellular elements of white matter. Further studies concerning the relative respiration of neural and glial cells of cerebral cortex became possible as a result of the antecedent experiments. Moreover a possible apportionment of the oxygen consumption of whole brain by its various components is suggested.

METHODS

The isolation and concentration of a neuroglial fraction and a myelin-rich, cell-poor fraction from lamb brain has been described in detail previously (KOREY *et al.*, 1958; KOREY, 1958). Briefly the method consists in sucking off the grey mantle and nuclear masses from the white matter of the centrum ovale. The latter is minced and blendorized carefully for 10 sec at slow speeds in 0·25 M-sucrose and 0·003 M-K_2HPO_3. The suspension is filtered through a siliconized silk grid and the residue resuspended. About 6–8 cycles are necessary to fractionate 30 g white matter. The suspension of white matter, filtered through another silk grid, is added as the uppermost layer to a 250 ml centrifuge tube containing an under layer of 1·75 M-sucrose, 0·003 M-K_2HPO_4 and a middle layer of 0·5 M-sucrose, 0·003 M-K_2HPO_4. After centrifugation at $500 \times g$ for 20 min the interface between the 0·5 M- and 1·75 M-sucrose layers is secured. This interface contains glial cells both free and attached to myelinated fragments, some red blood cells and endothelial components, and myelinated axonal fragments. Highly disrupted cells, their subcellular particles and the major amount of myelinated axons remain in the 0·25 M- and upper parts of the 0·5 M-sucrose layers.

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The desired interfacial layer is made up to 1·0 M-sucrose, relayered over a solution of 1·75 M-sucrose and centrifuged at 18,400 × g for 15 min. A thick myelinated axonal cell-poor layer rises to the top of the 1·0 M-layer while the cellular concentrate remains at the interface between the 1·0 M- and 1·75 M-sucrose layers. Both fractions are removed and dispersed in Krebs Ringer for respiratory studies. All procedures are carried out at 0–4° C.

About 0·5–1 ml of packed cells could be obtained from the white matter of twenty lamb brains. Each sample was examined microscopically before use. The appearance of these fractions has been discussed before (KOREY *et al.*, 1958).

Both fractions, the myelin–axon rich and the cellular concentrate, were homogenized in a Potter–Elvehjem homogenizer in Krebs-Ringer saline buffered with phosphate pH 7·4 at 0–2°. Native cortex and white matter were similarly treated. A 5–10% homogenate was used for studies of oxygen consumption.

Because of the washing procedures necessary to obtain the several fractions it was thought likely that many essential cofactors would be lost during the processes of concentration and isolation. Since this was found to be the case the homogenates of the fractions as well as the cortex and white matter were investigated in detail with regard to their requirements for maximal oxygen consumption and a supplemented media was developed. Moreover previous studies indicated that the substrate combination found to promote greatest respiratory activity in homogenates of white matter was succinate and pyruvate. In grey matter the addition of glutamate to these produced maximal respiration (KOREY *et al.*, 1958).

The respiratory studies were carried out at 37° in the Warburg apparatus. Substrates were placed in the side arms and a filter paper wetted with 0·2 ml 20% KOH was placed in the centre well. After thermal equilibrium was achieved the substrates were tipped into the main compartment of the vessels. Readings were taken at 5 min intervals. The rate of oxygen consumption remained steady for 30–40 min but gradually decreased in the case of the cellular concentrates. The values obtained for the first 20 min of incubation were used in the following calculations.

Purified coenzymes were obtained commercially. Adenosine triphosphate (ATP) in potassium form; coenzyme-A (CoA) and diphosphopyridine nucleotide (DPN) from Pabst; cytochrome-C from Sigma. Substrates were obtained from Nutritional Biochemicals and with the exception of sodium pyruvate were used without further purification. Sodium pyruvate was purified as its lithium salt.

Proteins were precipitated in 10% trichloracetic acid and washed twice. Nitrogen (Ntca) was determined by micro-Kjeldahl procedure. Nucleotides were analysed according to LOGAN, MANNELL and ROSSITOR (1952).

RESULTS AND DISCUSSION

In the present system the oxygen consumption of 1 g (wet wt.) corpus callosum was 1465 μl O_2/hr. Previous studies summarized in Table 1 indicated that one-third of the total respiration of white matter may be attributed to axoplasmic constituents. Thus in the corpus callosum 488 μl O_2 may be considered axoplasmic and 977 μl O_2 non-neuronal in origin. Accurate data for the number of cells present in corpus callosum and other areas of the central nervous system recently have been obtained in several mammalian species by NURNBERGER and GORDON (1957). For example cell

counts in the striate cortex of rat, monkey and man indicate that the neuronal population in this region is 15, 13·8 and 18·5 per cent respectively of the total cells. Moreover, the variations in the numbers of non-neuronal cells from species to species with

TABLE 1

Tissue	RESPIRATION (μl O_2/hr)		Ratio
	per 1 μM-RNA-P	per cell	
Neuroglial concentrate	1535	$11{\cdot}5 \times 10^{-6}$	
'Myelin' fraction			
Total	710		
Axoplasmic	557		
Cellular	153		
Axoplasmic/total			0·78
Cellular/total			0·22
White matter		$9{\cdot}2 \times 10^{-6}$	
Total	895		
Axoplasmic	318		
Cellular	577		
Axoplasmic/total			0·35
Cellular/total			0·65

Oxygen consumption by homogenates of neuroglial concentrates (N.C.); myelinated axonal fraction, (M.F.); and white matter (W.M.) of lamb brains. M.F. contains $7{\cdot}6 \times 10^5$ cells/mg Ntca. W.M. has $6{\cdot}36 \times 10^6$ cells/mg Ntca. M.F. is very similar to W.N. in RNA-P content, lipid distribution and weight per milligram. Ntca.

Thus with reference to Ntca: $\dfrac{\text{RNA-P}_{\text{W.M.}} - \text{RNA-P}_{\text{M.F.}}}{\text{Cell No.}_{\text{W.M.}} - \text{Cell no.}_{\text{M.F.}}} \cong$ RNA-P per non-neuronal cell,

or 0·24 $\mu\mu$g. If all RNA-P in W.M. is extra-axoplasmic, the non-neuronal cell would contain 0·47 $\mu\mu$g. Thus the amount 0·24 $\mu\mu$g/cell appears in likely range. Respiration of tissue fractions are referred to RNA-P as a cytoplasmic index.

In N.C.:

$$\text{respiration}_{\text{N.C.}} \cong \frac{\mu\text{l } O_2 \text{ per RNA-P}}{\text{no. of cells per RNA-P}} \cong 11{\cdot}5 \times 10^{-6}\ \mu\text{l } O_2\text{/hr per cell}$$

In M.F.:

$$\text{total respiration}_{\text{M.F.}} - (\text{no. cells in M.F.} \times 11{\cdot}5 \times 10^{-6}) = \text{axoplasmic respiration}_{\text{M.F.}}$$

In W.M.:

$$\frac{\text{total respiration}_{\text{W.M./mgNtca}} - \text{axoplasmic respiration}_{\text{W.M./mgNtca}}}{\text{no. cells}_{\text{W.M./mgNtca}}} =$$

$9{\cdot}2 \times 10^{-6}\ \mu$l O_2/hr. per cell

Axoplasmic respiration in W.M. was considered to be equivalent to that in an equal amount of M.F. using Ntca as referant for both. The similarity of the chemical and histological constitution of myelinated axonal tubules mentioned above supported this assumption. The conditions for studying respiratory activities of homogenates are described in Table 2. N.C. is a fraction containing chiefly glial perikaryons, fragments of glial processes, glial nuclei with their cytoplasmic halo and a few endothelial cells. M.F. consists in a concentrate of myelinated axons depleted of perikaryons (KOREY *et al.*, 1958; KOREY, 1958).

reference to grams (wet wt.) of cortical tissue are very limited, e.g. 8×10^7 in albino rat, $8{\cdot}88 \times 10^7$ in *macaca rhesus*, $7{\cdot}63 \times 10^7$ in man. We have applied these estimates for cell densities to this study using as references $7{\cdot}8 \times 10^7$ non-neuronal cells/g (w/w)

corpus callosum; $9{\cdot}7 \times 10^7$ cells/g (w/w) cerebral cortex of which $2{\cdot}3 \times 10^7$ are neuronal and $7{\cdot}4 \times 10^7$ non-neuronal cells. In the case of the corpus callosum where the non-axoplasmic respiration is 977 μl O_2/hr per g (w/w) and the cell density is $7{\cdot}8 \times 10^7$/g (w/w) the average non-neuronal cell respires at the rate of $12{\cdot}5 \times 10^{-6}$ μl O_2/hr. This value is to be compared with $11{\cdot}5 \times 10^{-6}$ μl O_2/hr per cell of the neuroglial concentrate and $9{\cdot}2 \times 10^{-6}$ μl O_2/hr per cell of whole white matter. The difference between 9·2 and 12·5, although suggestive, cannot be said to reflect differences between the more mixed population of non-neuronal cells found in white matter of centrum ovale and the glial population of the corpus callosum which is more oligodendroglial in type. The values may represent experimental variation magnified by calculation.

If the assumption can be made that the average non-neuronal cell respiration in the cerebral cortex is similar to that of corpus callosum the average neuronal cell oxygen consumption can be calculated. There are two major objections to this assumption: (1) the non-neuronal population in the cerebral cortex is composed of greater numbers of endothelial cells and microglia than are present in the corpus callosum; and (2) the true glial elements of the cortex including the satellites may have dissimilar respiratory rates to the glia of white matter. On the other hand by assuming that the oxygen consumption of the non-neuronal cells of cortex is $12{\cdot}5 \times 10^{-6}$ μl O_2/hr per cell we are applying the greatest value for respiration by non-neuronal cells we have obtained and presumably are minimizing the contributions of neurones to the total cortical respiration. At present the necessary data to overcome both these objections are not available.

Cerebral cortex of lamb brain (1 g w/w) respires 4147 μl O_2/hr as determined with the fortified homogenate system. Non-neuronal cell respiration in the cortex is equivalent to: $(12{\cdot}5 \times 10^{-6})$ $(7{\cdot}4 \times 10^7) = 925$ μl O_2/hr per g (w/w). Neuronal respiration is: $4147 - 925 = 3222$ μl O_2/hr per g (w/w). Since there are $2{\cdot}3 \times 10^7$ neurones/g (w/w), the average neuronal respiration is 140×10^{-6} μl/hr/per cell. It must be realized that this includes the contribution of axoplasmic constituents of fibres originating both in cortical and extra-cortical neurones. An attempt may be made to estimate the amount of axoplasm in the cortex to see whether or not its respiration is a significant proportion of the total. The proteolipids are believed to be derived from myelinated axons and can be employed as an indirect and very approximate estimate of the axonal material present. The ratio of proteolipids between grey and white matter is about 1:5 (FOLCH-PI and LEBARON, 1958). Thus it is possible that 1 g of fresh cerebral cortex could contain the equivalent of 0·2 g axoplasm. Since the axoplasmic respiration of 1 g of white matter (e.g. corpus callosum is one-third of its total respiration or about 450 μl/hr), 0·2 g of myelinated axons may account for about 90 μl O_2/hr, truly an insignificant contribution. In following through with this type of calculation one should consider that the cellular compartments of 1 g fresh cerebral cortex would be reduced to 0·8 g. On this basis the average neurone's respiration would be 136×10^{-6} μl O_2/hr. Since many axons in the cerebral cortex are said to be relatively unmyelinated the ratio of proteolipids between grey and white matter may not reflect the proportion of axoplasm present in cerebral cortex. However if instead of 0·2 g axonal material there were 0·4 g present in each gram (w/w) cerebral cortex, the axoplasmic contribution would be 180 μl O_2/hr and the average neuronal oxygen consumption 132×10^{-6} μl O_2/hr per cell,

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values not significantly different from those already expressed. In any event nerve cells account for about three-quarters of the respiration in cerebral cortex and are much more active in this respect than the average non-neuronal cell in white matter.

Analysis of ELLIOTT and HELLER's (1958) data regarding the respiration of the cerebral cortex reveals that they have greatly overestimated the proportion of neurones in the cerebral cortex. Recalculation of their data, which are the most pertinent in the literature, according to their method is given as follows: their stated assumption is similar to ours namely that the average non-neuronal cell in the cerebral cortex respires at the same rate as the average cell in the corpus callosum. The percentage, P, of the total respiration of cerebral cortex which is due to neurones is: $P = \{1 - f(rg/rt)\} \times 100$ where f is the ratio of non-neuronal cells to the total number of cells in the tissue; rt is the respiration rate of the average cell of all types (19×10^{-6} μl/hr); rg is the respiration rate per average non-neuronal cell ($5{\cdot}7 \times 10^{-6}$ μl/hr) P is found to be 83 per cent assuming that $f = 0{\cdot}5$. Thus if 83 per cent of the respiration of the cortex is due to neurones and half the cells are neurones the respiration rate of the average cortical neurone is 32×10^{-6} However realizing that the estimated f, the proportion of non-neuronal cells to total cell population, is too low one may substitute the average values derived from NURNBERGER and GORDON's (1958) data.

On this basis

$$P = \left(1 - 0{\cdot}77\,\frac{5{\cdot}7 \times 10^{-6}}{19 \times 10^{-6}}\right) \times 100$$

$$P = 77 \text{ per cent}$$

Moreover since 77 per cent of the total cortical respiration is calculated as neuronal and only 23 per cent of the cells are neuronal, the respiratory rate of the average neurone is 63×10^{-6} μl/hr about twice that calculated originally by the authors. Further the relative respiratory rates of the neurones and the non-neuronal cell of the corpus callosum are about 11:1, a factor quite similar to our observations. The fact that in ELLIOTT and HELLER's experiments the maximal respiratory rates both of the neurone and non-neuronal cell are half those we have measured in the homogenate system is in keeping with the trend in experimental values for Q_{O_2} in the two laboratories. The Q_{O_2}'s obtained by HELLER and ELLIOTT (1958) with brain slices were 12–13 for cortex and 2·2–2·7 for white matter while our similar determinations using fortified homogenates were 24·4 and 5·2 respectively. In their studies, the neuronal and non-neuronal cells utilized 63×10^{-6} and $5{\cdot}7 \times 10^{-6}$ μl O_2/cell per hr and in the present report 136×10^{-6} and $12{\cdot}5 \times 10^{-6}$ μl O_2/cell per hr respectively.

The values obtained by MCILWAIN (1955) for respiration in separated cortical tissue in an unfortified glucose saline medium subjected to electrical pulses are intermediate in amount, in the order of 2500 μl O_2/g (w/w)per hr. Table 2 summarizes the results of these experiments.

It is difficult in any case to compare *in vitro* experiments with the results furnished by the KETY technique *in vivo*. Nevertheless a qualified attempt may be made to translate the values obtained in these studies to human brain for the purpose of establishing the potential degree of respiration in neuronal and glial cells. There is reasonable quantitative justification for this (ELLIOTT, 1955). In a 1400 g brain there is about 650 g of grey matter (SHOLL, 1956) including cortex, basal ganglia, other nuclear masses and scattered cellular groups assumed to be respiring at similar rates.

TABLE 2

Tissue	Respiration (hr) Q_{O_2}	μl O_{O_2} (per g w/w)	μl $O_2 \times 10^{-6}$/cell Neuronal	μl $O_2 \times 10^{-6}$/cell Non-neuronal	No. of cells $\times 10^7$ (per g w/w) Total	Neuronal	Non-neuronal	Respiration (%) Neuronal	Non-neuronal	'Axoplasmic'
Cerebral cortex	24·4	4147	136	12·5	9·7	2·3	7·4	76	22	2·0
Corpus callosum	5·2	1465		12·5	7·8		7·8		67	33

The respiration of homogenates of cortex and corpus callosum and neuroglial concentrates derived from white matter of the centrum ovale and corpus callosum of lamb brains was measured in an incubation medium containing in final concentration: K_3ATP 1×10^{-3}; DPN 6×10^{-4}; cytochrome-*c* 3×10^{-5}; $MgCl_2$ 1×10^{-2}; KF 1×10^{-2}; K-succinate 1×10^{-3}; Na pyruvate 1×10^{-3}; and in the case of cortex K_2 glutamate 1×10^{-3}. A volume of 1 ml of a 5–10% homogenate in Krebs–Ringer phosphate buffer pH 74 was added to 2·0 ml of the reactants (temp. 37°). The number of cells per gram (w/w) is taken from NURNEERGER and GORDON (1957). g(w/w) is the fresh weight of the tissue employed. Q_{O_2} is the μl of O_2 utilized per hr per mg dry weight.

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The remainder, 750 g, is white matter. The Q_{O_2} of brain based on our experimental values is:

$$\left.\begin{aligned} 650 \times 4150 &= 26{\cdot}9 \times 10^5 \\ 750 \times 1340^* &= 10{\cdot}0 \times 10^5 \end{aligned}\right\} = 36{\cdot}9 \times 10^5 \ \mu l\ O_2/hr \text{ per } 1400 \text{ g}$$

$$\frac{36{\cdot}9 \times 10^5}{1{\cdot}4 \times 10^3} = 26{\cdot}3 \times 10^2 \ \mu l\ O_2/hr \text{ per g (w/w) brain}$$

The dry weight of whole brain is about 23 per cent of the wet weight (ROSSITER, 1955).

$$2630/230 = 11{\cdot}4\ Q_{O_2}$$

The Q_{O_2} 11·4 corresponds to that obtained by KETY (1948) in adults, Q_{O_2} 9·9 and is less than that measured by KENNEDY (1956) in children Q_{O_2} 14–15. The magnitude of the Q_{O_2} does not suggest that under the conditions and within the brief experimental period a maximal rate of respiration has been achieved. For example in experiments with monkeys in a 'resting' state the Q_{O_2} was 11·1 but in the convulsive state, 19·5 (SCHMITT *et al.*, 1945). In accepting the general trend of these results in monkeys the ratio (convulsive Q_{O_2}/resting Q_{O_2}) may be applied to the present instance, in which case the Q_{O_2} could rise to 20·0 during a seizure state. If all elements of the central nervous system were affected equally by its excited state the average neurone would respire 239×10^{-6} μl O_2/hr and the average non-neuronal cell 22×10^{-6} μl O_2/hr. However if the non-neuronal cells were relatively inert, at least initially, to the convulsive state but a proportionate rise in 'axoplasmic' respiration occurred, the average neurone would respire at the rate of 300×10^{-6} μl O_2/hr; 86 per cent of cortical respiration would be due to nerve cells.

Further when the brain is viewed in its entirety with regard to oxygen consumption neuronal respiration remains quantitatively dominant. In Table 3 is presented the

TABLE 3.—THE APPROXIMATE PROPORTION OF OXYGEN CONSUMPTION CARRIED OUT BY THE NEURONAL, NON-NEURONAL AND AXOPLASMIC COMPONENTS OF GREY AND WHITE MATTER (TISSUE) AND THE PERCENTAGE OF TOTAL BRAIN RESPIRATION ATTRIBUTABLE TO EACH COMPONENT

Tissue and tissue components	Respiration ($\mu lO_2 \times 10^5$/hr)		Percentage	
	Tissue	Component	Of total brain	Of tissue
Grey matter	26·9		72·9	
Neuronal		20·4		76
Non-neuronal		5·9		22
Axoplasmic		0·6		2
White matter	10·0		27·1	
Non-neuronal		6·7		67
Axoplasmic		3·3		33
Total brain	36·9		100	
Neuronal		20·4	55	
Non-neuronal		12·6	34	
Axoplasmic		3·9	11	

* 1340 is the value obtained for the bulk of white matter (KOREY *et al.*, 1958).

distribution of respiration attributed to the various components of grey and white matter in the 1400 g brain and in 'resting' condition.

The magnitude of the oxygen consumption by the neuronal cell whether judged by the results of the experiments with homogenates or slices deserves attention. In both instances neuronal respiration amounts to 75 per cent of cortical respiration. Indeed a comparison between the oxygen consumption of the neurone and the biosynthetically active hepatic parenchymal cell emphasizes the respiratory activity of the neurone. According to studies by KERBS and JOHNSON (1948) the Q_{O_2}'s of rat cortex and liver slices are comparable and of the order of 13. On a cellular basis the rat liver is from two to two and a half times more dense than cerebral cortex per gram (w/w) and about 60 per cent of all cells of the liver are parenchymal in type (NURNBERGER and GORDON, 1957). If we ignore the contribution to respiration of non-parenchymal cells, each true hepatic cell respires at the rate of about 21×10^{-6} μl O_2/hr whereas with the same set of experimental data the corresponding rate for an average neuronal cell is 87×10^{-6} μl O_2/hr. In view of the very active metabolism of hepatic cells the significantly greater oxygen consumption by neurones requires an attempt at explanation. It is possible to conjecture that cortical respiration should be related to the surface area of neurones rather than cell density. On this basis neurones would have a considerably reduced respiration in comparison with hepatic parenchymal cells. Moreover by implication oxygen consumption of neurones would be concerned with phases of reformation and maintenance of the membrane about synapses and to a lesser extent along fibres.

Finally it should be stated that the partitioning of respiration according to neurones, non-neurones and axoplasm is artificial. Axoplasm respiration is essentially part of neuronal respiration. Indeed, despite the fact that there are mitochondria within the axons part of the respiration rendered as axoplasmic may be performed by subcellular particles in the extremities of processes of glial cells thought to make up the myelin sheath and included in our fractionation as axoplasmic.

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SUMMARY

Non-neuronal cells, chiefly neuroglial, and a myelinated axonal fraction poor in cells were obtained by separation and sedimentation procedures from centrum ovale of lamb brain. Oxygen consumption of these components was measured in a fortified homogenate system. On the basis of these studies it was found that non-neuronal cells of the corpus callosum respired at the rate of $12{\cdot}5 \times 10^{-6}$ μl O_2/hr per cell. While the axoplasmic constituents of white matter accounted for one-third of the respiration, the non-neuronal cells were responsible for the remaining two-thirds. Cortical respiration was studied and the ratio of neuronal to non-neuronal respiratory rates were calculated to be about 11:1. It was possible likewise to approximate the contributions of grey and white matter, neurone, non-neurone and axoplasm to the respiratory activity of the entire brain.

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IMPORTANT EDITORIAL ANNOUNCEMENT

In 1959 the Journal of Neurochemistry will pass to regular bi-monthly publication, Full-length papers will be published within five months from their date of acceptance by the Editors; preliminary communications will appear in sixty to ninety days from their date of acceptance.

Owing to the increase in the number of excellent manuscripts accepted for publication in the Autumn of 1958, the publishers will issue Number 4 of Volume 3 in February 1959, and Numbers 1 and 2 of Volume 4 will appear in March and April 1959 respectively.

Submission of Manuscripts. The Editors wish to point out that they welcome the submission of papers or preliminary communications in the pharmacological and physiological fields, as well as that of biochemistry.

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INTRALAMINAR DISTRIBUTION OF ADENOSINETRIPHOSPHATASE ACTIVITY IN RAT CEREBRAL CORTEX*

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IN recent years, the principles and techniques of quantitative histochemistry have been used for a number of investigations on the biochemical architecture of the cerebral cortex. The principle object has been to provide detailed information on the chemical structure and metabolism of this tissue that could be related to its microscopic anatomy and electrophysiological properties. To this end, studies have been reported on the intralaminar architectonic distributions of enzymes in rat isocortex (POPE, 1952; POPE, HESS, WARE and THOMSON, 1956), rabbit hippocampus (LOWRY, ROBERTS, LEINER, WU, FARR and ALBERS, 1954), monkey motor and visual cortices (ROBINS, SMITH, EYDT and MCCAMAN, 1956*b*) and human frontal cortex (POPE, CAVENESS and LIVINGSTON, 1952; POPE, HESS and ALLEN, 1957), as well as of certain structural components in rabbit (LOWRY *et al.*, 1954) and in monkey cortex (ROBINS, SMITH and EYDT, 1956*a*).

Details of the experimental design and rationale for studies being carried out in this laboratory on the quantitative histochemistry of rat somatosensory isocortex have been described previously (POPE, 1952; POPE *et al.*, 1956). One of the enzymatic actions, the quantitative distribution of which has been investigated, is the hydrolysis of adenosinetriphosphate† under different conditions of assay. The studies of ENGELHARDT and LJUBIMOWA (1939) first showed ATPase activity to be a property of myosin, thus suggesting that enzymatic activity of this sort represents a special mechanism for energy transformation, although the details of this process have not been fully elucidated. The biochemical and physiological significance of ATPase activity in brain is even less clear, but it probably can be regarded as an index of the potential rate of adenosine polyphosphate metabolism.

Prior to 1950, several investigations had suggested a specific function for ATPase activity in nervous tissues. POTTER, SCHNEIDER and LIEBL (1954), measuring Ca–ATPase activity in the whole brains of developing rats, and FLEXNER and FLEXNER (1948), studying apyrase activity in the frontal cortices of foetal and adult guinea pigs, found that the enzyme activity in immature brains rose from low levels to adult values within a short and well demarcated time interval. FLEXNER and FLEXNER (1948) pointed out the correspondence of this rise in ATPase activity with important morphological changes in the cortex. LIBET (1948) showed that in the giant axon of the squid

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† The following abbreviations are used: ATP for adenosinetriphosphate; Ca–ATPase and Mg–ATPase for adenosinetriphosphatase activity stimulated by Ca^{++} and Mg^{++} respectively; AChE for acetylcholinesterase; TCA for trichloroacetic acid; and tris for tris (hydroxymethyl) aminomethane.

Ca–ATPase activity, like that of AChE (BOELL and NACHMANSOHN, 1940), is many times higher in the sheath than in the extruded axoplasm. Preliminary assays of Ca–ATPase activity in rat somatosensory cortex showed it to have an intracortical distribution closely similar to that of AChE (POPE, WARE, and THOMSON, 1950) and consistent with its localization in axonal and dendritic plexuses. LIBET suggested, on the basis of his study of squid nerve, that Ca–ATPase might be important in nerve impulse conduction.

Meanwhile, MEYERHOF and WILSON (1947), UTTER (1950), and GORE (1951) demonstrated that in brain tissue the activation of ATP breakdown by Mg^{++} exceeded that by Ca^{++}, and BINKLEY and OLSON (1950) isolated from mammalian brain a Mg–ATPase similar to that obtained from muscle by KIELLEY and MEYERHOF (1948). Since this work seemed to indicate that separate Ca^{++}- and Mg^{++}-activated enzymes were present in brain, the further study of ATP hydrolysis in rat somatosensory cortex was carried out using two assay systems, one incorporating Ca^{++}, the other Mg^{++} as activating ion. Preliminary presentation of this work has been made (HESS and POPE, 1954) and, in this article, the nature and outcome of the experiments are described in full. Divergent intracortical distribution patterns have been obtained under the two assay conditions, in accord with the conclusion that separate enzymes, having different cytological locations and different physiological roles, are being studied.

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MATERIAL AND METHODS

Sherman strain albino rats of both sexes were sacrificed by decapitation. The brains were removed without delay and frozen in a dry ice box. Before sampling, the brain was allowed to stand for 20 min in a cold room at $-12°$, following which a block of cortex was cut from the somatosensory area of one hemisphere. A metal cork borer, having an internal diameter of 2 mm, was used to punch from this block of cortex a cylinder whose long axis was perpendicular to the pial surface. The cylinder was mounted on a rotary microtome and a series of sections, 20 μ in thickness, were cut from it, progressing from the pial surface through the cortex into white matter. The sections were utilized in consecutive groups of four slices each, the first slice for fixation, staining (Nissl) and histological study; the second for assay of Ca–ATPase; the third for desiccation and weighing by means of an ultramicro quartz balance of the type described by LOWRY (1944); and the fourth for assay of Mg–ATPase. In addition, vertical histological sections were prepared from the region of cortex immediately adjacent to the site of the cylinder and stained with reduced silver. In these, the thicknesses of the cortical layers were measured directly. By comparing the measured thicknesses with the architectonic levels of the horizontal histological control series, it was possible to estimate reasonably accurately the thicknesses of the cytoarchitectonic layers in each individual cylinder (POPE, 1952).

Ca^{++} and Mg^{++} stimulated ATPase activities, expressed in terms of the weight of the appropriate desiccated section, were plotted for each experiment as a function of the theoretical depths of the respective samples beneath the pial surface and in relation to estimated thicknesses of the cytoarchitectonic layers. In this way, the cytoarchitectonic distribution patterns of both enzymes were established in each experimental cylinder. Since the total cortical thicknesses were of the order of 1600 to 1700 μ and the samples for each ATPase series were taken at 80 μ intervals, the results of each experiment provided data on both Ca–ATPase and Mg–ATPase activities at twenty or more evenly spaced intervals from pial surface to subjacent white matter.

Enzyme assays. Ca–ATPase and Mg–ATPase activities were measured using incubation media the constituents of which were essentially identical except for the divalent cation. In preliminary assays for Ca–ATPase, a micro-adaptation of the method of DUBOIS and POTTER (1943) was utilized. This technique, however, was unsatisfactory for studying Mg–ATPase activity because homogenization resulted in a loss of from one-half to two-thirds of the activity obtainable from intact micro slices. On the other hand, Ca–ATPase activity was similar in homogenates and slices. Consequently, both were assayed in relatively intact slices.

Each freshly cut frozen section to be assayed was placed in a precooled micro-test tube. The slice

was allowed to melt and adhere to the bottom of the tube. The series of samples obtained in each experiment was desiccated over $CaCl_2$ for 2 hr or longer at 4° before substrate solution was added.

The substrate solutions for Ca–ATPase assays contained the following final concentrations of reagents: 0·005 M-Na_2ATP; 0·030 M-NaCl; 0·1 M-glycylglycine, pH 7·4; and 0·003 M-$CaCl_2$. For the corresponding Mg–ATPase assay medium, 0·007 M-KCl was included, and 0·007 M-$MgCl_2$ (or $MgSO_4$) was substituted for $CaCl_2$. The assays for Ca–ATPase and Mg–ATPase utilized 30 and 60 μl of substrate solution respectively. These volumes provided an excess of ATP such that slices of 15–20 μg dry weight catalysed the enzymatic release of phosphorus equivalent to 10–15 per cent of the terminal phosphate of ATP during a 15 min incubation period. In the more active Mg–ATPase reaction, the release of phosphorus was a linear function of incubation time up to 2 hr, when the equivalent of 45 per cent of the terminal phosphate had been released. There was, therefore, no evidence for inhibition of the reaction up to this point by the reaction product, ADP. Accumulation of ADP may well have been prevented by the action of adenylate kinase.

Ice-cold substrate solution was pipetted into the sample tube and the slice was broken up and freed from the bottom of the tube by means of a fine hollow glass stirring rod. The tube was tapped to disperse the comminuted tissue throughout the medium, covered tightly, and incubated immediately for exactly 15 min at 37° in an oven. The reaction was terminated by immersing the sample tube in a freezing mixture of dry ice and acetone. Zero time control samples were included with each group of assays.

Enzyme inactivation and protein precipitation were brought about by trichloroacetic acid. For each 30 μl volume of substrate solution, 5 μl of 50% TCA were added to the frozen sample, which was thawed and mixed; this was followed by a volume of 7% TCA (usually 50–70 μl) which was sufficient both to dilute the ATP concentration* to 1–2 mM and to ensure that the optical density readings would be within the range covered by the phosphorus standards. The diluted sample was mixed by 'buzzing' (Bessey, Lowry, Brock and Lopez, 1946) and centrifuged for 12 min at 2000 rev/min in an International clinical centrifuge at 4°, or for 5 min at 4500 rev/min in a cooled microcentrifuge.† A 50 μl aliquot of the supernatant was transferred to a microtube for phosphorus analysis. Because of the lability of ATP and ADP in 7% TCA at room temperature and at 0°, the acid aliquot was either analysed promptly or stored on dry ice for a period not longer than 2 days.

Inorganic orthophosphate was determined by a micro-adaptation of the method of Fiske and Subbarow (1925), using the Beckman DU spectrophotometer equipped for microtechniques as described by Lowry and Bessey (1946). The fused quartz microcuvettes had a height of 25 mm, a light path of 10 mm and a window 1·5 or 3·0 mm wide. The Fiske and Subbarow reagents were added to the 50 μl aliquot of sample in the following order: 36 μl of water, 10 μl of 2·5% ammonium molybdate in 3 N-H_2SO_4 and 4 μl of 0·125% aminonaphtholsulphonic acid.‡ The solution was mixed by 'buzzing' transferred to a microcuvette, and allowed to stand exactly 15 min at room temperature for full colour development. The optical density of samples and standards was read at 410 mμ.

Reagents. Reagents for enzyme assays were made up in glass-redistilled water. Two preparations of disodium ATP were used. One was a 90 per cent pure, non-chromatographed salt from Sigma Chemical Company, the chief impurity of which was ADP. The other, a crystalline chromatographically prepared salt from Pabst Laboratories, contained very little ADP and was free of non-adenine nucleotides. The stock solutions of ATP and Sigma glycylglycine were neutralized to pH 7·4 (determined with a Beckman one-drop electrode) by the addition of crystals of tris, and stored frozen. Eastman Kodak technical grade aminonaphtholsulphonic acid and Sigma buffer grade tris were recrystallized.

RESULTS

The results of experiments in which the distributions of both Ca–ATPase and Mg–ATPase activities were determined in the cytoarchitectonic layers of the somatosensory cortices of eight rats are presented in tabular and graphical forms. Listed in Tables 1 and 2 are the values obtained for Ca–ATPase and Mg–ATPase activities

* Higher ATP concentrations in the usual Fiske and Subbarow test system interfere with the test by forming a colourless complex with the molybdate.

† Microcentrifuge No. 5500, Microchemical Specialties Company.

‡ The concentration of aminonaphtholsulphonic acid was lowered to 0·125 per cent to avoid the turbidity which otherwise tended to occur in low phosphorus samples and in the blank.

respectively in each cortex at successive, regularly spaced, subpial levels. Horizontal lines indicate to which cortical layer each sample was assigned on the basis of the histological control material for each experiment. In each table, the same data have been combined in two different ways by recognizing two types of sample groups in the population. A series group comprises all slices having the same subpial depth, regardless of their histological assignment. A layer group is made up of all samples belonging to a single cytoarchitectonic layer or sublayer, as identified by the histological control material. In Fig. 1, the series means for both sets of experiments have been plotted so as to bring out graphically the intracortical profiles of Ca–ATPase and Mg–ATPase in relation to the classical cytoarchitectonic structure.

The reasons for analysing the data in terms of both series and layer groups and the meaning of the large standard errors of the layer means require some discussion. The

TABLE 1.—CA-ATPASE ACTIVITY IN RELATION TO CYTOARCHITECTURE OF RAT SOMATOSENSORY ISOCORTEX

Series No.	Depth μ	Layer	Ca–ATPase activity (μmoles P released/mg dry wt. per hr)										
			Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Rat 8	Series Mean	Layer Mean	Layer SEM
1	50	I	3·86		2·38		3·72	3·69	4·67	1·47	3·30	3·34	0·43
2	130		1·99	1·95	2·80	4·73	3·63	3·85	4·94	1·59	3·19		
3	210	II	2·43	2·18	2·23	3·55	2·10	3·12	3·40	1·82	2·61	2·69	0·30
4	290	a	2·00	2·03	1·61	3·03	2·89	3·25	3·87	1·86	2·57	2·75	0·24
5	370	III	2·50	1·96	2·75	3·11	3·56	3·01	3·56	2·21	2·83		
6	450	b	2·23	1·37	2·08	2·53	3·11	3·43	4·04	1·76	2·57	2·60	0·30
7	530		2·07	1·86	2·04	3·12	3·41	2·89	3·89		2·75		
8	610	IV	2·12	1·05		2·22	2·94	3·06	3·87	1·37	2·38	2·30	0·36
9	690		1·94	1·33	2·22	2·07	2·05	2·67	4·12	1·69	2·26		
10	770	a	1·82	1·58	0·81	2·94	2·62	2·19	3·94	1·51	2·18	2·23	0·28
11	850		1·63	1·34	2·10	2·38	1·92	2·47		1·90	1·85		
12	930	V b	1·34	1·01	1·90	2·10	2·78	2·36	2·83	1·39	1·96	1·98	0·30
13	1010		1·15	0·32	1·45	2·16	2·53	3·14	3·38	1·64	1·97		
14	1090	c	1·16	1·48	1·90	1·70	3·24	2·09	3·61	1·32	2·06	1·99	0·32
15	1170		1·15	0·84	1·52	1·88		2·01	3·27	1·01	1·67		
16	1250		1·51	0·57	1·39	2·05	1·78		3·36	1·19	1·69		
17	1330	a	1·54	1·60	2·17		2·04	1·43	3·16	1·24	1·88	1·68	0·20
18	1410		1·50	1·15		2·12	1·82	1·30	2·13	1·03	1·58		
19	1490	VI	1·08	0·92	2·07	1·74		1·38	2·48	0·92	1·51		
20	1570	b	1·02	0·90	1·27		2·29			0·85	1·25	1·16	0·13
21	1650		0·70	0·89	1·46	0·93	1·48	0·67	2·14	0·92	1·15		
22	1730		0·77	0·83	1·25	1·10	0·84	1·28	1·51	0·79	1·05		
23	1810		0·76		1·27	1·17			1·58	0·70	1·10		
24	1890	WM			1·03	0·88			1·45	0·67	1·01		
Avg. for cortex			1·66	1·29	1·87	2·33	2·54	2·63	3·41	1·40			

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TABLE 2.—MG-ATPASE ACTIVITY IN RELATION TO CYTOARCHITECTURE OF RAT SOMATOSENSORY ISOCORTEX

Series No.	Depth μ	Layer	Mg-ATPase activity (μmoles P released/mg dry wt. per hr)										
			Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Rat 8	Series Mean	Layer Mean	Layer SEM
1	90	I	9·71	8·80	7·23		8·66	7·75	9·33	6·52	8·29	8·29	0·42
2	170	II a	7·97	10·32	9·39	11·72	8·97	8·49	14·46		10·19	10·19	0·84
3	250	II b	9·17	8·40	7·42	10·84	7·75	8·20	11·06	7·97	8·85	8·85	0·49
4	330	III a	9·48	9·00	8·51	7·73	8·52	8·17	12·91	9·98	9·29	9·35	0·52
5	410	III a	9·26	12·61	7·19	8·73	9·72	9·53	11·31	7·00	9·42		
6	490	III b	7·73	9·59		7·59	9·31	10·24	10·73	10·77	9·42	9·34	0·33
7	570	III b	9·91	9·24	10·44	8·47	9·30	8·24	11·05	8·93	9·48		
8	650	IV	11·62	7·86	10·43	7·49	9·76	9·42	12·85	10·43	9·98	9·90	0·56
9	730	V a	8·06	9·51	8·25	8·46	7·76	8·73	10·93	11·28	9·12		
10	810	V a	12·17	8·08	5·22	8·87	7·84	6·21	9·76	11·68	8·73	8·88	0·61
11	890	V a	7·36	8·42	7·11	7·68	8·61	7·53		12·91	8·52		
12	970	V b	8·29	7·62	8·31	8·02	7·77	7·66	9·30	6·95	7·99	8·15	0·41
13	1050	V b	6·97	9·84	6·96	6·88	8·65	8·83	12·21	7·42	8·47		
14	1130	V c	8·89	7·90	4·91	5·99	9·67	6·21	13·68	6·64	7·97	8·56	0·75
15	1210	V c	6·82	8·07	7·73	7·26		6·38	11·15	12·28	8·53		
16	1290	VI a	6·39	8·62	5·33	6·78	7·50		11·26	9·01	7·84		
17	1370	VI a	3·94	7·49	8·00		10·10	5·89	9·10	6·97	7·36	7·65	0·52
18	1450	VI a	7·81	6·78		7·41	9·33	4·63	9·77	6·71	7·49		
19	1530	VI a	6·36	6·55	7·23	6·73		4·89	7·75	10·73	7·18		
20	1610	VI b	3·09	5·62	5·87		6·11			6·24	5·55		
21	1690	VI b	3·15	5·46	6·15	5·13	3·51	2·25	7·53	3·42	4·58	4·88	0·30
22	1770	VI b	4·02	4·54	4·26	3·69	2·89	2·81	5·69	3·61	3·94		
23	1850	WM	4·98		3·24	2·49			7·17		4·47		
24	1930	WM			1·74	1·94			3·91		2·53		
Avg. for cortex			7·55	8·20	7·30	7·66	8·09	7·61	10·59	8·45			

use of the series groups allows greater objectivity in interpreting the data. First, it is not based upon the presupposition that division of the cortex into six layers of neuronal cell bodies has unique significance in relation to the distribution of biochemical constituents. Second, it is independent of the subjective judgement of the investigator regarding the assignment of the sample to the cortical layers and sublayers. While many biochemical components may be primarily related to the cortical layers, it is also possible that some constituents are more closely associated with anatomical features other than neuronal cell bodies, such as plexuses of axons and dendrites. Such plexuses, poorly visible by the usual methods of tissue sampling and histological control study, undoubtedly have important biochemical properties. The constancy of their relationship to the visible neuronal population within a given area of cortex is a very approximate one only (LORENTE DE NÓ, 1943). The possible influences of such

structures upon the biochemical architectural pattern might be obscured when the results are considered in cytoarchitectonic terms only.

However, because most investigations upon the anatomy and biochemistry of the cortex have utilized the six-layered cytoarchitectonic pattern as a reference system, the results have also been expressed in terms of the classical cytoarchitecture. For each experiment, all values assignable to the commonly used layers and sublayers were

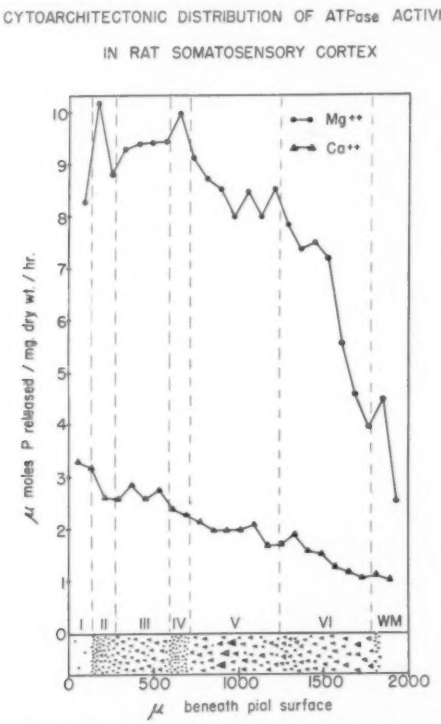

FIG. 1. Intralaminar distribution patterns of Ca–ATPase and Mg–ATPase activities in rat somatosensory isocortex. Cytoarchitectonic features as revealed by the Nissl stain and average thicknesses of the cortical layers are indicated along the abscissa.

averaged. The mean and its standard error were then calculated for each set of layer and sublayer averages for the entire series of experiments. The layer means thus arrived at and their standard errors are shown in columns 13 and 14 of Tables 1 and 2. These data provide some indication of the intraspecies means and ranges of activities of Ca–ATPase and Mg–ATPase within the cytoarchitectonic layers and sublayers of rat somaesthetic cortex.

The meaning of the large standard errors and the variability of presumably comparable results from animal to animal have been discussed previously in connexion with findings on other enzymes (POPE *et al.*, 1956), but repetition and further comment are in order. This variability is not due to analytical inaccuracy, since the standard deviation of the microchemical procedures themselves is $\pm 3\%$ when successive samples of uniform histological composition are analysed. Rather, it is the result of inherent intraspecies variations in ATPase activities, as demonstrated by the spread of mean values for cortex and white matter as a whole, and of the lack of exact reproducibility

in the sampling procedures employed in these experiments. Since the experimental design provides serial samples at standard, successive intracortical depths, the architectonic composition of a given serial sample in different experiments will vary in accordance both with the total cortical thickness and the widths of the constituent layers and sublayers in the individual animal, and with the exactness of orientation of the particular cortical cylinder during the cutting of the serial microtome sections. For these reasons, it is not surprising that considerable variations in ATPase activities are found in corresponding serial sections from experiment to experiment, and that even when the results are averaged in terms of particular cytoarchitectonic landmarks, the standard errors of these means are large. These factors limit the value of statistical treatment of the results, although it is clear that averages for given serial samples in a group of such experiments must approach a normal value for the intracortical level in question.

Because of the foregoing difficulties, a very simple type of analysis has been applied to the results of these, as of other similar experiments (POPE, 1952; POPE *et al.*, 1956). This has consisted in comparing the intralaminar ATPase distribution pattern in each individual experiment with that of the corresponding composite curve as shown in Fig 1, and noting the frequencies with which the characteristics of the composite pattern were repeated among the individual experiments. In this way, recurring and characteristic features of the intralaminar distributions of Ca–ATPase and Mg–ATPase can be satisfactorily ascertained in relation to the cytoarchitectonic structures. In Fig. 1, the series means are plotted as a function of the subpial depth. The average thicknesses of the cytoarchitectonic layers and the character of the neuronal population in the somatosensory cortex are indicated along the abscissa. The distributions of Ca–ATPase and Mg–ATPase activities are shown on the same scale to make their differences readily apparent. In cortical grey matter, the average Ca–ATPase activity (2·14 μmoles P released per mg dry wt. per hr, S.E.M. 0·68) is only one fourth of the average Mg–ATPase activity (8·18 μmoles P released per mg dry wt. per hr, S.E.M. 0·99). Activity in subcortical white matter was only one-fourth to one-third as great as the maximum activity in cortical grey matter in the case of both enzymes.

The pattern of Ca–ATPase activity illustrated at the bottom of Fig. 1 is characterized by high activity in layer I, a general tendency to decreasing activity as a function of the subpial depth, and small peaks of activity in sublayers IIIa, IIIb, Vc and VIa. These features were typical of the individual experiments. The values were highest in layers I, IIa and III. In five of the eight experiments, layer I had the greatest activity. In two of the remaining experiments, the maximum of activity was found in layer IIa, while in one it was in layer III. Values in the lower part of layer II were appreciably less than those in layer I in six of the eight experiments. In each of the eight cortices, layer III showed either a single or a double peak of activity, second in magnitude only to the activity in layer I or IIa. In six of the seven experiments in which layer IV was sampled, the average Ca–ATPase activity was less than and, in one experiment, it was about equal to the average activity in layer II. Although in four of seven instances activity in layer Va was greater than in the neighbouring slice of layer IV, no peak appeared in layer Va in the composite curve. The peak in the lower part of layer V was more pronounced and was seen in seven of eight experiments; this peak was usually only a little lower than values in layer Va and in two instances it was nearly as great as were the peaks in layer III. In the seven experiments where sampling of layer VI was

complete, layer VIa had significantly higher mean activity than did VIb. White matter was sampled in only half of the experiments. In these, the activity of immediate subcortical white matter was slightly less than that in the lower part of layer VIb.

The intralaminar distribution of Mg–ATPase activity, shown at the top of Fig. 1, has features which distinguish it from that of Ca–ATPase activity. Mg–ATPase activity was more consistently elevated in layers II, III and IV than elsewhere, and was never greater in layer I than in layer IV. The higher activity in layer IV was a much more constant characteristic of the individual experiments (7 of 8) than was that in layer III. The site of peak activity in layer III was variable, so that although many of the values in this layer were higher than those in layers II and IV, the average values for a given depth were lower. Activity in layer V showed considerable variability; its most constant feature was an elevation in layer Vb, or more frequently in Vc, which was present in all experiments. In half of the experiments, spikes of activity in layer Va equalled or exceeded the activity in the remainder of layer V. Mg–ATPase activity was higher in layer VIa than in VIb and declined through VIb to low values in white matter.

In brief, the results show that Ca–ATPase activity is maximal in layer I and at the I–IIa border; thereafter, it gradually declines as a function of increasing depth beneath the pial surface, although relatively greater activity is found in sublayers IIIa, IIIb, Vc and VIa. On the other hand, Mg–ATPase activity is highest in layers II, III and IV, especially in IIa and IV; in addition, a small peak of activity occurs in sublayers Vb or Vc as well as in VIa. A comparison of the layer means for Ca–ATPase and Mg–ATPase, listed in Tables 1 and 2, or an inspection of the composite curves in Fig. 1 reveals not only that the enzyme patterns are different, but that for the most part the variations tend to be inverse. In particular, this tendency is seen in layers I and IV.

DISCUSSION

In previous quantitative histochemical studies of enzymes and coenzymes in rat somatosensory isocortex, several distinct intracortical distribution patterns have been encountered which could be correlated with certain features of the anatomical fine structure. The histological information required for these correlations has been obtained by examining and measuring the parallel histological preparations and by referring to classical investigations upon the cytoarchitecture and myeloarchitecture (CAJAL, 1911; LORENTE DE NÓ, 1922, 1943; KRIEG, 1946) and the vascular supply (CRAIGIE, 1921; CAMPBELL, 1939) of somatosensory cortex in different mammals, many pertinent details of which were summarized in a preceding article (POPE, 1952).

The anatomical localization of Ca–ATPase will be discussed by trying to assess, in turn, the part played by neuroglial, vascular and neuronal elements in determining the intracortical pattern.

While oligodendrocytes and astrocytes may have some degree of Ca–ATPase activity, their influence upon the intracortical distribution pattern is probably small. This seems likely for two reasons. First, their numbers per unit volume are relatively constant from layer to layer in the cortex, except for some increase in astrocytes in layers I and IV and of oligodendroglia in layer VI. In contrast, Ca–ATPase activity is notably lower in layers IV and VI than in neighbouring layers. Second, although white matter contains greater numbers of neuroglia per unit volume than does cortex, its Ca–ATPase activity is only one-fourth as high.

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The evaluation of the importance of vascular ATPase as a contributor to the intracortical pattern of Ca–ATPase is more difficult. Since the smooth muscle layer of blood vessels contains actomyosin, Ca–ATPase activity may be elevated wherever muscular vessels are found. NAIDOO and PRATT (1956) separated the cortical surface vessels from rat cerebral hemisphere and reported the vascular Ca–ATPase activity, in terms of fresh weight, to be tenfold that of the remaining ectodermal tissue. In the present experiments, the most obvious site for the occurrence of this type of vessel is in the first slice of layer I, centred 50 μ beneath the pial surface. Inspection of the layer means in Table 1 reveals that the overall cortical mean of activity is exceeded by 56 per cent in layer I, by 25 per cent in layers II and III, and by 8 per cent in layer IV; while the activities in layers V and VI lie below the cortical mean. For muscular vessels to be responsible for the increment of activity in slice 1, nearly 15 per cent of the dry weight would have to be of vascular origin.* Although at times a small cresent of pia mater might have been included in this slice, the presence of so large a fraction of vascular material is doubtful; and this explanation for the equally high values in slice 2, centred 130 μ below the pia, is even less likely. On the other hand, at least some of the Ca–ATPase activity found in layer I probably can be ascribed to vessels. However, muscular vessels are not known to be distributed at other levels in a pattern similar to that of Ca–ATPase, and smaller vessels differ by showing the greatest density in layer IV (CRAIGIE, 1921).

The distinguishing feature of the intracortical distribution profile of Ca–ATPase activity is its resemblance to that previously observed for AChE activity (POPE, 1952). AChE activity is greatest in layer I, at the junction of layers II and IIIa, and in layers IIIb, Va and Vc. These peaks of activity coincide with zones which are relatively acellular in Nissl-stained sections and contain dense plexuses of axones and dendrites. This correlation suggests that AChE activity is principally associated with the neuronal processes and may be localized in the finer subdivisions of dendrites and axones, perhaps at their surfaces and synaptic junctions. The intracortical profile of Ca–ATPase is like that of AChE in exhibiting peaks of activity in layers I, IIIa, IIIb and Vc, but it shows relatively less decline in activity in layer IV. The approximate parallelism of the two intralaminar distributions suggests that Ca–ATPase, like AChE, may be principally localized in cortical axonal and dendritic plexuses. In this connexion, it is of some interest that both were also localized in the sheath fraction of squid giant fibres (BOELL and NACHMANSOHN, 1940; LIBET, 1948). The neurophysiological significance, if any, of this association between Ca–ATPase and AChE activities will be decided only by more precise determinations of their histochemical localizations and better understanding of their respective metabolic roles. Recent reports that preparations of AChE (BERNHARD, 1955) show ATPase activity and that myosin has AChE activity (VARGA *et al.*, 1955) are noteworthy; but, beyond the fact that they imply some similarity of binding sites on the two macromolecules, their meaning is not clear.

The apparent localization of Ca–ATPase activity in axonal and dendritic plexuses in cerebral cortex is a point in favour of an involvement of an actomyosin-like protein

* The average value for Ca–ATPase activity in cerebral cortex in the present series of experiments was 2·14 μmole of P released per mg dry wt. per hr, and the average value for the first slice was 3·30. The activity for surface vessels obtained by NAIDOO and PRATT (1956) was about 3430 μmoles of P per g fresh wt. per hr. If the dry weight of vessels is about 30 per cent, vessel Ca–ATPase is then 11·4 μmoles of P per mg dry wt. per hr. Calculations from these values indicate that 12·5 per cent of the dry weight must be vessel in order to raise the parenchymal value to 3·30.

in the permeability changes coincident with depolarization in nervous structures, as suggested by LIBET (1948). Possible mechanisms by which contractile surface protein might govern permeability and secretion processes in cells have been discussed by GOLDACRE (1952), DANIELLI (1952) and BURGEN (1957). However, to speculate upon the mechanical details of such a process in relation to the neurone seems unprofitable at this time. When the interactions of actomyosin, Mg^{++}, ATP and Ca^{++} have been further clarified by the study of muscle, the model may serve to illuminate permeability processes in nervous tissues.

The intracortical distribution pattern of Mg–ATPase activity is similar to that previously described for cytochrome oxidase, but the patterns differ in certain details. Cytochrome oxidase activity is fairly uniform within layers I to V, although, relative to neighbouring layers, greater activity occurs in sublayers IIa, mid-III, and Vb or Vc. Oxidase activity rapidly declines through layer VI to values in subcortical white matter which are only one-fourth of the highest values in gray matter. This intracortical distribution is consistent with localization of cytochrome oxidase in neuronal perikarya, large dendrites, and axone terminals (POPE *et al.*, 1956). Since cytochrome oxidase is exclusively a mitochondrial enzyme, its intracortical profile may be considered, in fact, a direct portrayal of the mitochondrial population at different cortical levels.

Mg–ATPase activity follows, in general, the pattern shown by cytochrome oxidase, but its height in layers IIa and IV tends to exceed that in layer III, while oxidase activity in layers IIa and III is greater than in IV. The distribution of Mg–ATPase thus corresponds more nearly than does that of the oxidase with the neuronal cell body population and with the capillary density (CRAIGIE, 1921), both of which are greatest in layer IV. It seems probable that the parallel between Mg–ATPase activity and the vascular supply reflects the relation of both to the intensity of oxidative metabolism during activity and that capillaries themselves do not contribute greatly to ATPase activity. The slight differences between Mg–ATPase and cytochrome oxidase distributions suggest that the former activity may be more closely associated with neuronal cell bodies, especially with those having rich axosomatic terminals, although dendrites and their connexions are also major sites of activity. In white matter, Mg–ATPase, like cytochrome oxidase, has an activity which is only one-fourth of its maximum in the grey matter. A similar relation between Mg–ATPase activity in monkey cortex and underlying white matter has been reported by ROBINS *et al.* (1956*b*).

Analogous architectonic studies of Mg-ATPase activity in rabbit hippocampus (LOWRY *et al.*, 1954) and in the motor and visual cortices of the monkey (ROBINS *et al.*, 1956*b*) are difficult to compare closely with the present results, not only because the areas of cortex sampled were not the same in the three animal species, but also because somewhat different assay conditions were used. In general, however, the findings are not at variance when the differences in anatomical fine structure of the cortical areas are considered. For example, the lesser activity of layer IV in monkey motor cortex may perhaps be explained by its sparse population of neurones in comparison with the analogous layer in rat parietal cortex. It is notable that the 33 per cent greater activity of Mg–ATPase reported in monkey visual cortex as compared with motor cortex accompanies a much higher neuronal cell density in the former.

The resemblance of the intracortical distribution of Mg–ATPase to that of cytochrome oxidase is consistent with the occurrence of the former, also, in intracellular

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particulates, but probably not exclusively in mitochondria. In fact, differential centrifugation studies indicate the presence of both Ca–ATPase and Mg–ATPase activities in the particulate fractions; however, the interpretation of this work in terms of localization of activity in cellular organelles is difficult.*

It has been suggested that mitochondrial ATPase activity may be due, at least in part, to the disordered function of one or more enzymes participating in oxidative phosphorylation (HUNTER, 1951; KIELLEY and KIELLEY, 1951). HÜLSMANN and SLATER (1957) have described four ATPases in liver and heart mitochondria, three of which are stimulated by dinitrophenol (and by Mg) and may be related to specific phosphorylating steps in the respiratory chain. The fourth enzyme, unaffected by dinitrophenol, was activated by Mg^{++}. The significance of this Mg–ATPase activity and that occurring in microsome sized particles is not known. Although appearing to be hydrolytic, its true function may be transphosphorylative. The Mg–ATPase activity measured by microassay as described in this article may thus be a product of two types of enzymes, one located in mitochondria, the other elsewhere, perhaps in the cell surface or in endoplasmic reticulum.

The Mg–ATPase activity of microslices of cortex has been reported to show characteristic responses to changes in the concentrations of NaCl and KCl (HESS and POPE, 1957). This work will be described in detail elsewhere. Analogous effects of sodium and potassium ions upon a Mg–ATPase separated from crab nerve led SKOU (1957) to suggest that the enzyme might play a part in the active transport of sodium ions. PAPPIUS and ELLIOTT (1954) also expressed the idea that Mg–ATPase activity might be related to the regulation of intracellular concentrations of ions. An indirect control over this process would obviously be exerted by an enzyme showing the type of ATPase activity which may represent a reversal of a phosphorylation step in the respiratory chain, since its normal function would help to maintain the ATP needed for active transport processes. Whether nervous tissues contain, in addition, a Mg–ATPase which is more directly related to the active transport of sodium ions, remains to be determined.

SUMMARY

The distributions of Ca–ATPase and Mg–ATPase activities have been studied in the cytoarchitectonic layers of rat somatosensory cortex.

The intracortical distribution of Ca–ATPase activity shows a maximum in layer I, subsidiary peaks in layers IIIa, IIIb, Vc and VIa, and a general tendency to decline in activity with increasing subpial depth. The general pattern is consistent with the localization of Ca–ATPase activity in the cortical plexuses of axones and dendrites,

* Differential centrifugation studies, using both cerebrum (JORDAN and MARCH, 1956) and nerve (ABOOD and GERARD, 1954; SKOU, 1957) seem to indicate that the highest percentage of ATPase activity is located in particles 1 μ and less in diameter. This would include particles usually designated mitochondria, as well as microsomes. The specific activity in terms of N content, however, was appreciably greater in the smaller particles. The Mg–ATPase preparations of KIELLEY and MEYERHOF (1948) and of SKOU (1957) were obtained as microsome sized particles. It seems probable that in addition to Mg–ATPase present in latent form in mitochondria and capable of being activated under adverse physical and chemical conditions, nervous tissues contain a non-latent Mg–ATPase in structures which give rise to microsome-sized particles in the differential centrifugation procedure. Unfortunately, this technique does not distinguish between localization of an enzyme in the endoplasmic reticulum (intracytoplasmic membranes) and in the cell surface, since both membranes probably disintegrate into particles of a similar size. Moreover, apparently the Nissl body component characteristic of the neuronal endoplasmic reticulum may fragment into multiple-sized particles (WAKED and KERR, 1955), thus further complicating anatomical correlations in this technique. The fact that Ca–ATPase and Mg–ATPase activities show similar distributions in brain tissue particulates (JORDAN and MARCH, 1956) does not prove their location in the same cell organelles.

although a part of the activity in layer I may be ascribed to blood vessel actomysin.

Intracortical Mg–ATPase activity is greatest in layers II, III and IV, while small secondary peaks tend to occur in layers Vb or Vc and in VIa. This pattern is consistent with localization of the activity chiefly in neuronal perikarya, large dendrites and axone terminals where cell particulates abound.

The divergent patterns obtained are in accord with the conclusion that in brain tissue Ca^{++} and Mg^{++} predominantly activate separate enzymes whose cytological locations and physiological roles are different.

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COMPOUNDS IN BRAIN EXTRACTS CAUSING SPREADING DEPRESSION OF CEREBRAL CORTICAL ACTIVITY AND CONTRACTION OF CRUSTACEAN MUSCLE

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DURING spreading depression (SD) the cortex passes through a cycle of major changes. The spontaneous electrical activity becomes deeply depressed, and the potentials evoked by sensory and electrical stimulation of the cortex are greatly reduced (LEÃO, 1944; MARSHALL, 1950; OCHS, 1958). Spreading depression is accompanied by a slow potential change usually consisting of a negative variation followed by positivity of the cortical surface with respect to an indifferent electrode (LEÃO, 1947, 1951; MARSHALL, ESSIG and DUBROFF, 1951; VAN HARREVELD and STAMM, 1951; BUREŠ, 1954). The electrical resistance of the cortex increases markedly (LEÃO and FERREIRA, 1953; FREYGANG and LANDAU, 1955; VAN HARREVELD and OCHS, 1957). A swelling of the apical dendrites by the uptake of water (and ions) from the intercellular spaces can be demonstrated (VAN HARREVELD, 1958).

Changes which are similar to those observed during SD develop with a latency of several minutes after circulatory arrest. The cortical surface becomes negative with respect to an indifferent electrode (LEÃO, 1947, 1951; VAN HARREVELD and STAMM, 1953). The time course of this potential is reminiscent of that of the slow potential change accompanying SD. Simultaneously the cortical resistance increases very markedly (VAN HARREVELD and OCHS, 1956) and a swelling of perikarya and apical dendrites takes place (VAN HARREVELD, 1957).

The neuronal changes during SD and after circulatory arrest seem to involve a large portion, if not all of the cortical neuronal elements. Such widespread changes may be caused by the release of a compound which then should be present in the cortex a few minutes after stopping the blood flow. To search for such compounds, extracts were prepared of cortex collected about 5 min after circulatory arrest. These extracts as such, and after purification, caused SD when applied to the cerebral cortex.

The effects of cortical extracts on a number of other physiological preparations were investigated. The application of extracts in relatively high dilutions caused contraction of crustacean muscle. This preparation proved to be very convenient for the assay of the physiological activity of cortical extracts. Its usefulness was enhanced by the finding that so many of the properties of the compound eliciting contractions in crustacean muscle parallel those of the substance causing SD that it was probable that an identical compound was involved in both activities. The present paper deals with the identification of this compound.

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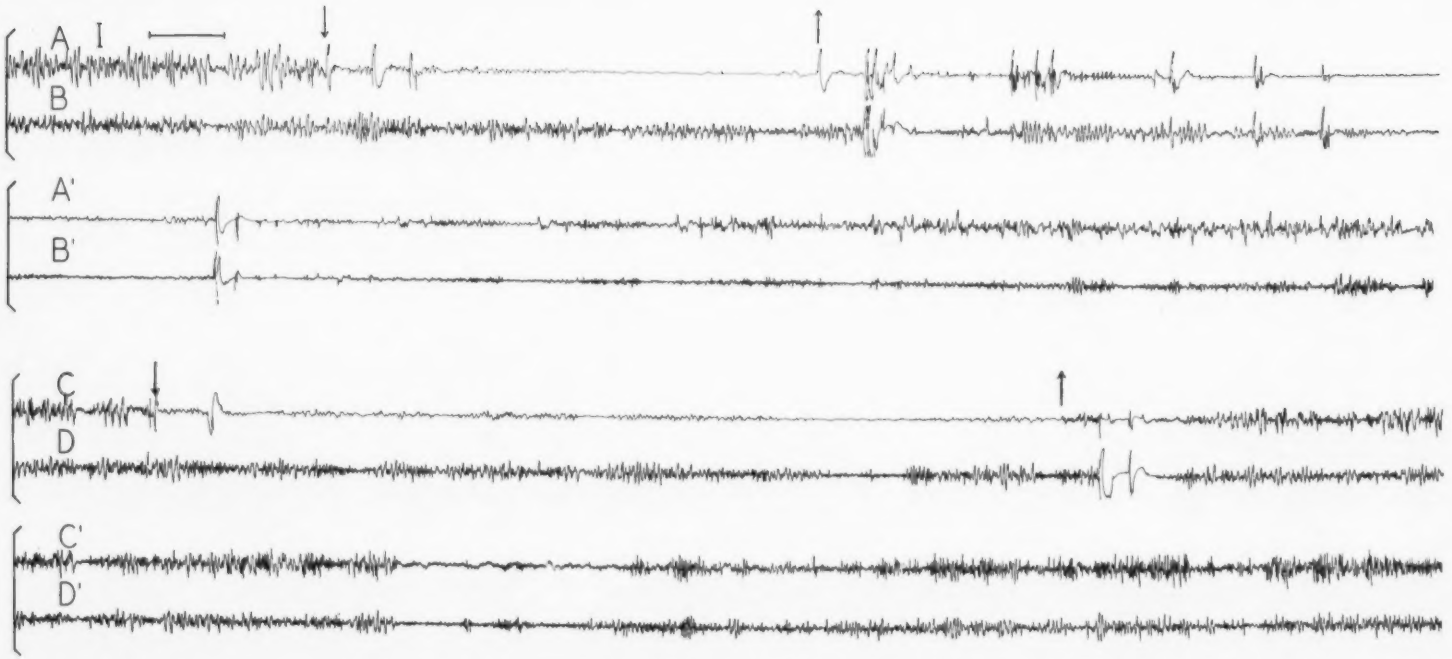

FIG. 1. Effect of L-glutamic acid applied to the cortex. The electrocorticogram *A* is continued in *A'*, *B* in *B'*, etc. The traces *A* and *B* are recorded from two electrode pairs placed on the convexity of the cortex; *C* and *D* are from the same pairs. *A* and *B* show the effects of the application of an effective, *C* and *D* of an ineffective concentration of L-glutamic acid. At the arrows pointing down, cotton pledgets moistened with the test solution are applied close to the electrode pair from which *A* and *C* are recorded. This produces artifacts, which are followed by others when additional drops of the test solution are applied to the pledget. The shorting of the electrodes decreases the amplitude of the corticogram in *A* and *C*. At the arrows pointing up the pledgets are removed and the cortex is flushed with Ringer's solution which is subsequently drained off. This caused more artifacts. In the experiment *A B*, L-glutamic acid is applied in a concentration of 0·013 moles/l. During the application the corticogram *A* flattens out. After removal of the pledget the depression deepens further; a few groups of spikes develop. In trace *A'* the cortex recovers. In trace *B*, *B'* the depression develops later and recovers later. In traces *C* and *D* taken from the same preparation L-glutamic acid is applied in the same way in a concentration of 0·006 moles/l. This concentration is below threshold and does not produce spreading depression. The sudden amplitude drops of short duration are arousal reactions. The vertical calibration line indicates 1 mv for both channels; the horizontal line indicates 5 sec.

METHODS

Rabbit cortex test preparation

The dorsal aspect of one hemisphere in young adult rabbits was exposed under ether narcosis. During the recording of the electrocorticogram the preparation was immobilized with Squibb's intocostrin (5 to 10 units/kg) and ventilated with a Palmer pump. Usually two pairs of electrodes were placed on the convexity of the hemisphere. The electrocorticogram was recorded with an Offner electroencephalograph. The test solutions were applied for a standard time of 2 min on small cotton pledgets which were placed close to one of the electrode pairs. Due to shorting of the leading-off electrodes this caused a decrease in amplitude of the electrocorticogram which made it sometimes

FIG. 2. Crustacean test preparation. Part of the fixed member of the pincer is cut off and a rubber tube clamped at one end is fitted over the remaining part. A hypodermic needle is stuck through the wall of the tube into the propodite. The test fluids and the physiological solution to wash them out are applied through the hypodermic needle. They flow out through a hole in the shell of the carpopodite.

hard to observe the depression in this channel. However, in such cases the development of a depression in the other channel after a period commensurate with the distance between the electrode pairs and the slow rate of propagation of SD (2–5 mm/min) showed that the application of the extract did elicit this phenomenon. Fig 1 shows two experiments, one in which the application of a test solution caused SD and one in which such an application failed to do so. It has been found by several authors (LEÃO, 1944; SLOAN and JASPER, 1950; WHIELDON and VAN HARREVELD, 1950) that during a series of SD's convulsive patterns tend to replace the depression. Since during an assay a series of SD's is elicited, such a transition can be expected and was regularly found. The response in Fig. 1 shows spikes which during subsequent 'depressions' developed into a full fledged convulsive pattern. A positive reaction after the application of a test solution thus may have been either a spreading depression or a spreading convulsion. In some preparations the application of brain extracts, or other test solutions normally active, failed to initiate SD, although this phenomenon could be produced by KCl in sufficient concentrations.

Crustacean muscle test preparation

The following simple preparation of the crayfish (*Cambarus clarkii*) was used for assaying purposes. One of the claws was removed by a cut through the ischiopodite. Half of the fixed member of the pincer was cut off and a short length of rubber tubing was snugly fitted on the remaining part. The free end of the tube was closed with a clamp and a hypodermic needle was introduced through its wall reaching into the propodite (Fig. 2). A hole was made in the shell of the carpopodite. Test solutions in amounts of 0·5 ml were forced with a tuberculin syringe through the needle. The test solution flowed through the muscle of the claw and ran out through the hole in the carpopodite. The test compounds could be washed out again by forcing crustacean physiological solution (VAN HARREVELD, 1936) through the claw.

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Extracts

(1) *Crude bulk extract.* The pallia* of from 20 to 40 rabbit brains (weighing 60–120 g) were frozen with dry ice about 5 min after decapitation, and ground in the frozen state. One ml 100% alcohol was added for each gram of brain tissue. The mixture was kept for 1 hr at 0°C with repeated shaking. It was then centrifuged and the supernatant fluid was dried *in vacuo* at about 40°C. The centrifugate was weighed. For testing on crustacean muscle the dried extract was dissolved in distilled water so that 1 ml of the solution contained the activity of 1 g of tissue.

For testing on the cerebral cortex, the dried extract of the 10 pallia of 5 brains (about 15 g of

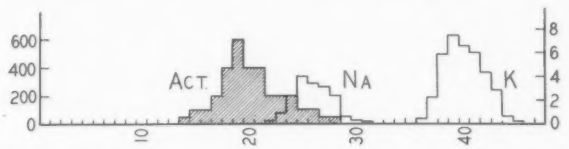

FIG. 3. The crude extract of 20 pallia (about 30 g of tissue) was adsorbed on a Dowex 50 column. After washing, the activity was eluted with 0·05 N-HCl. Fractions of 15 ml were collected. The 47 fractions obtained are indicated on the abscissa. The activity of each fraction, expressed in crustacean units, was determined and plotted on the ordinate (*left side*). Also the amount of Na and K expressed in mg per fraction was determined and plotted on the ordinate (*right side*).

tissue) was dissolved in 1 ml of distilled water. Dilutions with distilled water were made from this hypertonic solution which contained from 15 to 25 mg of potassium per ml. To remove some of the potassium a cation exchange resin (Dowex 50) was used. Since this resin also removed activity, it has to be applied with caution. When from 30 to 40 mg of Dowex 50 was used per gram of tissue there was little effect on the activity, whereas an appreciable amount of potassium was removed.

(2) *Purified extract.* The adsorption of the activity on Dowex 50 was used in a purification procedure. A column about 30 cm long and 0·6 cm wide was prepared with Dowex 50 in the hydrogen form (8 g). The extract of 20 pallia was passed through the column, which then was washed with 50 ml water. After this, the active compound was eluted with 0·05N-hydrochloric acid and fractions of 15 ml were collected. In each fraction the amount of sodium and potassium was determined with a flame spectrophotometer and the activity was determined with the crayfish preparation. As shown in Fig. 3 the activity passed through first, followed closely and partly overlapped by the sodium. Finally the potassium came through. This sequence allowed a complete separation of the activity from potassium. The separation between activity and sodium was not complete with a column of this length. The first fractions containing the active compound were free of sodium and by evaporating these *in vacuo* a sodium and potassium free extract could be obtained. Most of the hydrochloric acid used for elution was removed by evaporation *in vacuo*. The extracts were finally neutralized with sodium hydroxide. In all instances the sodium content and activity of the thus purified extract were determined and sodium, potassium and calcium ions were added to make a solution of the desired activity and of the ionic composition of Ringer's solution. The activity of the initial crude extracts was recovered in the purified extract without appreciable losses.

RESULTS

Effect on crustacean muscle

Activity of extracts of rabbit pallium assayed with the crustacean preparation. Pallium extracts elicit contractions of crayfish (*Cambarus clarkii*) muscle in relatively high dilutions. The maximum dilutions of extracts still causing muscle contraction were determined. The amount of active material in 1 ml of such a threshold dilution was designated as the crayfish unit. Crude extracts containing the activity of 1 g pallium/ml contained an average of about 200 units/ml. Test preparations showed considerable individual differences in sensitivity. Unknown test solutions were therefore always compared with the standard extract made of 20 to 40 pallia (see

* The pallium consists of the cortex with the underlying white matter.

Methods) in which differences in content of active material in individual pallia are averaged out.

Potassium ions in sufficient concentration also cause contraction of crustacean muscle (threshold concentration in 12 preparations was 0·03 ± 0·003 moles/l. or 1·2 mg K/ml). The crude pallium extract contained up to 2·0 mg K/ml. The first dilutions of extracts were made with potassium-free crustacean physiological solution until the potassium concentration was equal to, or below that of normal physiological solution (± 0·005 moles of K/l). Further dilutions were made with normal crayfish solution. This procedure eliminates the possibility that the muscle contractions observed would be due to potassium from the extracts.

Using the crustacean preparation to assay activity, the active compound was found to be heat resistant (boiling for 15 min). It was not oxidized by exposure to air at room temperature for 24 hr. It was not destroyed by bringing the extract at pH 3 or pH 12 for some hours. The active compound was not markedly adsorbed by carbon, but was completely adsorbed by a cation as well as by an anion exchange resin (Dowex 50 and Dowex 1). The activity could be eluted easily from the Dowex 50 column with dilute hydrochloric acid, but it was not found possible to recover the activity from a Dowex 1 column with ammonia in concentrations up to 20 per cent.

Chromatography and electrophoresis of pallium extracts. The adsorption of the activity both on cation and anion exchange resins suggested that the active compound might be an amphoteric electrolyte, for instance an amino acid. This possibility was investigated by chromatography of crude pallium extracts on filter paper. To obtain sufficient material for physiological testing a thick paper (Whatman no. 3), 50 cm long and 20 cm wide was used. The extract (0·2 to 1 ml) was applied almost over the whole length of the paper. The solvent used for ascending chromatography consisted of 2 vols. of *n*-propyl alcohol, and 1 vol of 1% (w/v) ammonia. Such chromatograms showed usually six separate bands of ninhydrin positive material. The paper was cut into strips which corresponded as much as possible with the bands and these strips were extracted with 70% (v/v) alcohol. After drying and dissolving in crustacean physiological solution the extracts were assayed with the crayfish preparation. Such experiments showed that the activity was mainly present in one strip. Chromatography of known amino acids with the same solvent suggested this to be the strip containing glutamic and aspartic acids which are incompletely separated with this solvent.

To establish further the identity of the active compound, small quantities of crude pallium extract (0·1–0·25 ml) were subjected to electrophoresis on filter paper. Spots of glutamic and aspartic acids served as identifying markers. An acetate buffer at pH 4 was used. The potential was 200–250 v, duration 4–5 hr. In this way a good separation of aspartic and glutamic acid is obtained. After thorough drying *in vacuo* over sodium hydroxide the paper was cut in strips taking care to include the glutamic and aspartic acid bands in different strips. The main, and often the only activity, assayed with the crayfish preparation was found in the glutamic acid band. Extracts of the aspartic acid strip were not active in several experiments. The electrophoresis experiments thus suggest that the compound in crude pallium extracts, causing contraction of crayfish muscle is glutamic acid.

In a second set of chromatographic and electrophoretic experiments the purified pallium extracts described under method were investigated. The chromatograms of

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'pure' extracts showed the same number of ninhydrin positive compounds. However, the glutamic acid band had become considerably darker as compared with the bands formed by the other compounds. After chromatography and electrophoresis the paper was again cut in strips and assayed with the crayfish preparation. The strips containing glutamic acid were often the only ones showing activity. It seems therefore that the fractionation process on Dowex 50 used in the purification favours glutamic acid as compared with other ninhydrin positive compounds, besides eliminating a number of compounds not absorbed on the resin.

Activity of some amino acids and related compounds examined with the crustacean preparation. As suggested by the chromatographic and electrophoretic experiments the effect of glutamic acid on the crustacean preparation was investigated. The naturally occurring L-isomer caused contractions in rather high dilutions. This amino acid and all other compounds investigated were neutralized and dissolved in crustacean physiological solution. The threshold dilution for contraction of L-glutamic acid was in most preparations between 0·05 and 0·025 m-moles/l. Table 1 gives as a mean of 12 experiments 0·035 m-moles/l. with a standard error of 0·003 m-moles/l. This corresponds to about 5 μg of L-glutamic acid per ml.

The stereoisomer D-glutamic acid up to a concentration of 20 m-moles/l. was without effect in 5 preparations. It caused muscle contractions in concentrations between 10 and 20 m-moles/l. in 3 claws. Two other dicarboxylic α-amino acids were investigated; of these L-α-aminoadipic acid* was without effect up to a concentration of 20 m-moles/l. Very slow contractions, developing much later than the reactions caused by L-glutamic acid, were elicited by L-aspartic acid at a mean concentration of 14 m-moles/l. Relatively active was L-glutamine which caused a contraction at a mean concentration of 2·4 m-moles/l.; L-asparagine was without effect in concentrations up to 20 m-moles/l. Glutaric and succinic acid, L-ornithine and L-proline had no effect. Other compounds tested were DL-α-aminobutyric acid, γ-aminobutyric acid, γ-keto-glutaric acid and ammonium chloride. All were without visible effect in concentrations up to 20 m-moles/l.

Comparison of the activity of extracts and of L-glutamic acid solutions. The minimum concentration of L-glutamic acid causing contraction of crustacean muscle was about 5 μg/ml. This makes it possible to answer the question whether crude pallium extracts contain sufficient quantities of this amino acid to explain their activity. Known amounts of pallium extracts in increasing dilutions were chromatographed on filter paper with the same amounts of L-glutamic acid and L-aspartic acid in known concentrations. Phenol was used for the development of the chromatograms (DENT, 1948; ROBERTS and FRANKEL, 1950), since this solvent separates glutamic and aspartic acid more completely than the *n*-propyl alcohol–ammonia mixture used in the earlier experiments. A comparison of the spots of known concentrations of the amino acids with those of the extract dilutions made it possible to make a crude estimate of the concentration of these compounds in the extract. The aspartic acid content found in this way was from 0·25 to 0·5 mg/ml of extracts containing the activity of 1 g of pallium per ml; the glutamic acid content was 1·5 to 1·75 mg/ml. However, phosphorylethanolamine and serine travel about as fast as glutamic acid on chromatograms developed with phenol. The presence of these compounds in an extract will make its glutamic acid content appear greater than it actually is.

* I am indebted to Drs. H. K. MITCHELL and P. H. LOWRY for two samples of this amino acid.

TABLE 1—THRESHOLD CONCENTRATIONS OF GLUTAMIC ACID AND OTHER COMPOUNDS (M-MOLES/L.) TESTED WITH THE CRUSTACEAN PREPARATION

Compound	Number of preparations	Threshold concentration (m-moles/l.)
L-Glutamic acid	12	0·035 ± 0·003
D-Glutamic acid	8	± 20
L-Aspartic acid	8	14 ± 2·4
L-α-Aminoadipic acid	5	—*
L-Glutamine	8	2·4 ± 0·2
L-Asparagine	4	—*
Glutaric acid	4	—*
Succinic acid	4	—*
L-Ornithine	4	—*
L-Proline	4	—*
DL-α-Aminobutyric acid	4	—*
β-Aminobutyric acid	4	—*
α-Ketoglutaric acid	4	—*
Ammonium chloride	4	—*

* The highest concentration in which these compounds were tested was 20 m-moles/l.

Two-dimensional chromatograms in which these three compounds can be identified separately were made as described by DENT (1948) and by ROBERTS and FRANKEL (1950). The serine spots in these were very light, but the phosphorylethanolamine spots had about one-fifth the intensity of the glutamic acid spots. The true concentration of glutamic acid thus can be estimated as 1·2 to 1·4 mg/ml. These figures agree well with those found by KREBS, EGGLESTON and HEMS (1951) and by TALLAN, MOORE and STEIN (1945) for grey matter and brain respectively. Since the activity of the crude extracts is usually about 200 units/ml and the mean L-glutamic acid equivalent of one unit is about 5 μg the extracts must contain approximately 1 mg of glutamic acid per ml if all the activity is to be ascribed to this amino acid. From these rough calculations it would seem that the crude extracts contain enough glutamic acid to account for their ability to cause contractions of crustacean muscle.

Effects on the cerebral cortex

Activity of pallium extracts assayed with the rabbit cortex preparation. In preliminary experiments the extract of 10 pallia (about 15 g of tissue), dissolved in 1 ml distilled water, was applied to the cortex on a small pledget of cotton; it promptly caused SD. However, KCl is a potent stimulus for SD and such extracts contain 15–25 mg K/ml (0·4 to 0·6 moles/l.). The mean threshold concentration of KCl solutions applied in the same way as the extract in eight experiments was 0·11 ± 0·01 moles/l.; the lowest threshold was 0·075 moles/l. BUREŠ (1956) found an SD 50 value of 0·08 moles/l. for KCl solutions applied to the cortex of the rat. In a search for compounds other than potassium capable of initiating SD, the extract was diluted until the potassium concentration was below threshold. In an occasional experiment SD was initiated by such diluted extracts, but in most instances this phenomenon could be produced only with extracts so concentrated that the potassium content was above threshold.

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It has been found previously (VAN HARREVELD and STAMM, 1955), that under the influence of Metrazol (pentamethylenetetrazol) the rabbit cortex tends to respond with SD to photic stimulation, a response rarely seen in preparations of this species not treated with this drug. Also minor convulsive patterns and perhaps even paroxysmal activity can in such preparations initiate the depression. Metrazol thus seems to facilitate the initiation of SD. It was attempted to sensitize the cortex with Metrazol for the assay of pallium extracts. To this end 0·20–0·25 g of Metrazol was slowly (in the course of about 10 min) injected intravenously. Developing convulsions were suppressed with small amounts (20–40 mg) of pentobarbital. After from 1 hr to 90 min the cortex of such preparations usually started to convulse spontaneously. This activity was often hard to control with pentobarbital without seriously depressing the spontaneous electrocorticogram. The time available for the assay of a brain extract was therefore limited. Since it takes from 5 to 10 min to test one dilution of an extract it was impossible to do many tests with one preparation. The assay of extract could for these reasons not be done as accurately as would be desirable.

In the majority of preparations treated with Metrazol SD was initiated with such dilutions of the crude pallium extract that the potassium concentration was below threshold for a KCl solution. The threshold test solutions in 10 such experiments contained on the average the activity of 1·4 g of pallial tissue per ml. The mean potassium concentration of these just active test solutions was 0·06 moles/l. In all the experiments the threshold concentration for a KCl solution was determined. The mean of these values was 0·11 $\pm$ 0·01 moles/l. It is of interest that the threshold concentration for potassium in Metrazol-treated preparations is the same as that found in the group of preparations mentioned above which had not been injected with this drug. These experiments indicate that there is present in pallium extracts an active principle other than potassium, which is able to initiate spreading depression.

Some of the properties of the compound producing SD were investigated using the rabbit cortex to assay activity. It was found to be heat resistant (boiling for 15 min). It was not oxidized by exposure to air at room temperature for 24 hr. The activity was not adsorbed by carbon, but was adsorbed both by Dowex 50 and Dowex 1. These properties resemble those of the substance causing contraction of crustacean muscle, suggesting that both activities are due to the same or similar compounds. The extracts purified by adsorption on a Dowex 50 column as described under method were quite potent in producing SD. This further supports the thesis that both activities are elicited by the same or similar compounds, since in the preparation of the purified extracts contraction of crustacean muscle was used as a guide for activity.

Table 2 shows the threshold concentration of purified extracts, which do not contain excess potassium, determined in 10 experiments after administration of Metrazol and in 10 experiments in which this drug had not been used. The potency of the extracts is expressed in crustacean units. The mean threshold concentration in the group of animals which had received Metrazol was 235 $\pm$ 34 units/ml. The mean threshold concentration in the untreated preparations was much greater, 1535 $\pm$ 253 units/ml. This difference is statistically highly significant ($t = 4{\cdot}1$; $P < 0{\cdot}01$). Metrazol thus lowers the threshold for the purified extracts markedly.

Activity of some amino acids and related compounds examined with the rabbit cortex preparation. The identification of the compound in the crude and purified extracts which causes contraction of crustacean muscle as glutamic acid suggested that this

TABLE 2—THRESHOLD CONCENTRATIONS OF PURIFIED EXTRACTS (EXPRESSED IN CRUSTACEAN UNITS) TESTED WITH RABBIT CORTEX

No.	0·2–0·25 g Metrazol (units/ml)	No.	No Metrazol (units/ml)
1	200	11	750
2	200	12	2250
3	375	13	800
4	200	14	2250
5	400	15	1600
6	100	16	2500
7	200	17	2500
8	100	18	800
9	375	19	400
10	200	20	1500
Means	235 ± 34		1535 ± 253

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TABLE 3—THRESHOLD CONCENTRATIONS OF L-GLUTAMIC ACID (IN M-MOLES/L.) TESTED WITH THE RABBIT CORTEX

No.	0·2–0·25 g Metrazol (m-moles/l.)	No.	No Metrazol (m-moles/l.)
1	5	11	10
2	2·5	12	20
3	5	13	10
4	10	14	10
5	5	15	25
6	2·5	16	13
7	10	17	25
8	5	18	5
9	5	19	25
10	5	20	10
Means	5·5 ± 0·8		15 ± 2·4

amino acid is also the substance initiating SD. Solutions of L-glutamic acid neutralized with sodium hydroxide and dissolved in mammalian Ringer's solution were applied to the cortex in the same way as described for the extracts. They were found to cause SD. Table 3 shows the threshold concentrations in 10 preparations which had been injected with Metrazol and 10 preparations to which this drug had not been administered. In the first group the threshold concentration varied between 2·5 and 10 m-moles/l.

The mean was 5·5 m-moles/l. with a standard error of 0·8 m-moles/l. The threshold in preparations which had not been treated with Metrazol was markedly higher, between 5 and 25 m-moles/l. The mean was 15 m-moles/l. with a standard error of 2·4 m-moles/l. The difference in threshold concentration of the two groups of preparations is statistically significant ($t = 3{\cdot}7$; $P < 0{\cdot}01$), indicating that Metrazol also sensitizes the cortex for L-glutamic acid. The threshold concentrations of 0·08 per cent and 0·22 per cent respectively, are low for this mode of administration of drugs to the cortex.

A number of compounds related to glutamic acid either chemically or metabolically were tested on their ability to produce SD. The threshold concentrations of those compounds which initiated SD are given in Table 4. In 5 preparations which had been injected with from 0·2 to 0·25 g Metrazol and in 5 preparations which had not been treated this way L-aspartic acid was assayed. The mean thresholds of 7 and 17 m-moles/l. respectively do not differ statistically from the corresponding values for L-glutamic acid. In 5 preparations not treated with Metrazol L-α-aminoadipic acid was tested. The mean threshold of 35 m-moles/l. may be significantly larger than that of L-glutamic acid in similar preparations. Surprising was the low threshold of D-glutamic acid. In 10 preparations which had not been treated with Metrazol the mean threshold was $3{\cdot}6 \pm 0{\cdot}5$ m-moles/l., which is lower than the threshold for L-glutamic acid in preparations which had been treated with Metrazol. As observed by BUREŠ (1956) L-glutamine was able to initiate SD, however, in the rather high mean concentration of 80 m-moles/l. Much more potent was L-asparagine which in 5 preparations caused SD with a mean threshold concentration of 13 m-moles/l. Of three dicarboxylic acids tested, only succinic acid caused SD in a concentration of 200 m-moles/l.; glutaric and α-ketoglutaric acid had no effect at this concentration. No higher concentrations than 200 m-moles/l. were tested since such solutions are already hypertonic. Spreading depression was elicited regularly with L-proline in a concentration of 100 m-moles/l.; L-ornithine was not active in concentrations up to 200 m-moles/l. Other compounds tested were DL-α-aminobutyric acid, γ-aminobutyric acid and glutathione; they were inactive in concentrations up to 200 m-moles/l. No SD was initiated by ammonium chloride in concentrations up to 100 m-moles/l., when applied in concentrations of 200 m-moles/l. this compound elicited convulsive activity in some preparations.

Comparison of the activity of extracts and of pure amino acids. The question can be asked whether the crude extracts contain enough of the active amino acids to account for their ability to cause spreading depression. In Metrazol treated preparations the threshold test solutions contained on the average the activity of 1·4 g of pallial tissue per ml as mentioned above. Chromatography of pallium extracts containing the activity of 1 g of tissue per ml showed that there was from 1·2 to 1·4 mg of glutamic acid and from 0·25 to 0·5 mg of aspartic acid in 1 ml of these extracts. These amino acids are equally active on the cortex (Tables 3 and 4). There was thus from 2·0 to 2·7 mg of the active amino acids per ml in the threshold test solutions. The mean threshold concentration of L-glutamic acid in Metrazol treated preparations was 5·5 m-moles/l. or 0·8 mg/ml. There is thus from two to three times as much glutamic and aspartic acid present in the crude pallium extracts as would follow from their activity. This discrepancy may be due to the presence in pallium extracts of compounds which inhibit the initiation of spreading depression. Indeed BUREŠ (1956) described an inhibiting effect of citric, α-ketoglutaric, succinic and malonic acid on

TABLE 4—THRESHOLD CONCENTRATIONS OF SOME COMPOUNDS (IN M-MOLES/L.) TESTED WITH THE RABBIT CORTEX

No.	0·2–0·25 g Metrazol (m-moles/l.)	No.	No Metrazol (m-moles/l.)
L-*Aspartic acid*			
1	10	6	20
2	5	7	10
3	10	8	10
4	5	9	25
5	5	10	20
Mean	7	Mean	17
L-α-*Aminoadipic acid* (*no Metrazol*)			
1	25	4	25
2	50	5	25
3	50		
		Mean	35
D-*Glutamic acid* (*no Metrazol*)			
1	3	6	5
2	2·5	7	1·3
3	5	8	2·5
4	5	9	5
5	1·3	10	5
		Mean	3·6 ± 0·5
L-*Glutamine* (*no Metrazol*)			
1	100	4	100
2	100	5	50
3	50		
		Mean	80
L-*Asparagine* (*no Metrazol*)			
1	13	4	6
2	10	5	10
3	25		
		Mean	13
Succinic acid (*no Metrazol*)			
1	200	3	200
2	200	4	200
		Mean	200
L-*Proline* (*no Metrazol*)			
1	100	3	100
2	100	4	100
		Mean	100

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the initiation of spreading depression by potassium chloride. However, the concentrated brain extracts contain so many compounds in such high concentrations that they may retard the diffusion of the active amino acids into the cortex, impeding the building up of the intracortical threshold concentration.

Effects on other preparations

The effects of pallium extracts and of L-glutamic acid were investigated on the blood pressure of the rabbit, the movements of rabbit intestine, the frog heart, blood vessels in the hind leg of the frog, and on crustacean peripheral nerve. The activity of none of these preparations was affected by L-glutamic acid. Conduction in crustacean nerve was arrested by crude, but not by purified extracts. It could be shown that this effect is due to the potassium content of the crude extracts. The crude extracts and in higher concentrations the purified extracts caused a drop in blood pressure, arrested intestinal movements, enhanced contractions and tone of the heart and caused vasoconstriction. These actions are not caused by excess potassium, since this is absent in the purified extracts, but they are caused by as yet unidentified compounds.

DISCUSSION

The effect of L-glutamic acid on crustacean muscle is a specific one. Contractions were evoked at a mean threshold concentration of 0·035 m-mole/l. None of the compounds related with this amino acid either chemically or metabolically caused this reaction in comparable concentrations. The most active of these compounds was L-glutamine which made the muscle contract at a seventy times higher mean concentration (2·4 m-moles/l.). The only other active compounds, L-aspartic and D-glutamic acid caused contractions in concentrations 400 to 600 times as high (14 and $\pm$ 20 m-moles/l.) as the threshold concentration of L-glutamic acid. The possibility that these active compounds were contaminated with L-glutamic acid cannot be excluded. Since glutamine tends to hydrolyse to glutamic acid, the results with this compound are particularly suspect even though the test solutions were stored under refrigeration and were frequently made up freshly. Nothing is known about the mechanism of the action of L-glutamic acid on the muscle. Since this amino acid did not have an obvious effect on crustacean nerve even in concentrations more than 1000 times those causing muscle contraction, it seems unlikely that L-glutamic acid elicits contractions by an action on the motor nerve. It probably acts on the neuromuscular junction or on the muscle fibre itself.

When applied to the cortex L-glutamic acid caused spreading depression in rabbits at a mean concentration of 15 m-moles/l. A number of compounds related chemically to L-glutamic acid were also able to elicit this phenomenon. About equal in potency were L-aspartic acid (17 m-moles/l.) and L-asparagine (13 m-moles/l.). The most active substance was D-glutamic acid which was about four times as active (3·6 m-moles/l.) as the above compounds. About half the potency of L-glutamic acid was found in experiments with L-α-aminoadipic acid (35 m-moles/l.). Some compounds able to elicit SD at higher concentrations were L-glutamine (80 m-moles/l.), L-proline (100 m-moles/l.) and succinic acid (200 m-moles/l.). Many compounds applied in a concentration of 200 m-moles/l. failed to elicit the depression. Among them were glutaric and α-ketoglutaric acid, L-ornithine, DL-α-aminobutyric and γ-aminobutyric acid. The latter amino acid blocks excitatory synapses in the cortex (PURPURA, GIRADO

and GRUNDFEST, 1957*a*). The same authors (1957*b*) found α-aminomonocarboxylic acids synaptically inactive.

It would seem possible that a compound like glutamic acid does not act directly, but that one of the products of its metabolism causes SD. This possibility is not supported by the findings, since the known product of the metabolism of glutamic acid, glutamine, α-ketoglutaric acid, α-aminobutyric acid and ammonia either do not elicit SD or do so at much higher concentrations than glutamic acid.

The test solutions were applied for a standard time of 2 minutes. During this time the active compound has to diffuse into the cortex to a locus where it can exert its action. However, since many of the compounds used are intermediary metabolites of the cortex, they may be changed into inactive or less active compounds. The rate of diffusion and the rate of these metabolic processes will determine the concentration reached at the place of action during the time the compound is applied. These considerations may explain some of the differences in threshold observed. One of the surprising findings was the high potency of D-glutamic acid as compared with its stereoisomer. In the cortex L-glutamic acid can participate in several reactions; it can be aminated to L-glutamine, by deamination it can be changed to α-ketoglutaric acid and finally it can be decarboxylated to γ-aminobutyric acid. It is unlikely that there are present in the cortex enzymes which can handle D-glutamic acid as effectively in the same way. Assuming that both stereoisomers diffuse into the cortex at the same rate, then the D-form will attain for this reason a higher concentration than the L-isomer. The higher potency of D-glutamic acid therefore does not mean necessarily that the process resulting in SD is activated by a lower concentration of this compound than of L-glutamic acid. It is even possible that this process is set in motion by a lower concentration of the L-isomer, but that the metabolic use of this compound hampers its accumulation to this concentration.

It was found that the administration of from 0·2 to 0·25 g of Metrazol enhances the sensitivity of the cortex for L-glutamic and L-aspartic acid from two to three times. Since the threshold concentration of KCl was the same in preparations which had been injected with Metrazol as in rabbits which had not been so treated, it seems unlikely that Metrazol increases the sensitivity of the neuronal elements involved in spreading depression. It is more likely that Metrazol inhibits the enzymic reactions which use glutamic and aspartic acid, thus allowing the building up of a higher concentration of these amino acids in the cortex.

The mean threshold concentration of the most active compound, D-glutamic acid was 3·6 m-moles/l. or about 0·05 per cent which is rather low for the application of drugs to the cortical surface. Spreading depression is probably a favourable phenomenon to be elicited in this way. There are indications that SD is propagated more readily in the surface layers of the cortex than in the deeper parts (LEÃO, 1944; LEÃO and MORISON, 1945; GRAFSTEIN, 1956*b*). Once SD is started in the surface layers which are the first to be reached by the test compounds the phenomenon is self-propagating and spreads over large regions of the cortex.

As to the structure of the active compounds, the four α-amino dicarboxylic acids investigated were all quite active (D- and L-glutamic, L-aspartic and L-α-aminoadipic acid). The length of the carbon chain does not seem to influence the activity greatly, although L-α-aminoadipic acid (6 C) was less active than L-glutamic (5 C) and L-aspartic acid (4 C). The replacement of a carboxyl group as in L-asparagine did not

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reduce the activity. The low potency of L-glutamine is surprising in this respect but may be due to a more ready use of this compound in the metabolism of the brain than L-asparagine. The removal of one of the carboxyl groups as in DL-α-aminobutyric acid or in L-ornithine makes the structure quite inactive. Unexpected in this respect is the moderate potency of L-proline. The elimination of the α-amino group also makes the compound inactive as in glutaric and α-ketoglutaric acid, or active only in very high concentrations as in succinic acid. BUREŠ (1956) found that the latter compounds inhibit the development of the spreading depression caused by the simultaneous application of KCl. No great specificity is apparent in the initiation of spreading depression; many compounds related to glutamic acid elicit this phenomenon. Also the finding that D- and L-glutamic acid both cause spreading depression clearly indicates that the specificity of this reaction is not great.

The concentrations of glutamic and aspartic acid needed to initiate SD at the locus of action in the cortex is not known but can be expected to be considerably lower than the 5·5 m-mole/l. in which L-glutamic acid acts in the Metrazol treated preparation. Extracts of pallium were found to contain from 1·2 to 1·4 mg glutamic-acid and from 0·25 to 0·5 mg aspartic acid per g of tissue, or from 8 to 10 m-moles and from 2 to 4 m-moles of these amino acids in 1000 g of pallium. As these compounds are intermediary metabolites they are probably mainly present inside the cellular elements where their concentrations can be expected to be higher than in the extracts. Since there is already so much glutamic and aspartic acid present in these structures it is not very likely that the application of these compounds in relatively low concentrations to the cortex as was done in the present experiments could materially affect the metabolism of these amino acids. This concept is strongly supported by the high potency of D-glutamic acid since, as mentioned above, it seems unlikely that there would be present in the cortex enzymes which could handle the D-isomer as effectively as the L-form. It would seem therefore that the compounds producing SD act by their presence in the intercellular fluid outside the neuronal elements.

A compound which is present in the cortex and is able to initiate SD could well be involved in the propagation of this phenomenon. GRAFSTEIN (1956*a*) paid considerable attention to this possibility. She recorded intense neuronal activity in the beginning of SD and postulated that this would cause the release of potassium from the active units, depolarizing adjacent cells and fibres which in turn would become active, releasing more potassium, etc. Indeed potassium chloride in relatively high concentration (0·11 moles/l.) elicits SD. However, potassium ions are physiologically quite active and have a variety of non-specific effects. Compounds with a more specific action might be involved in the mechanism of SD and of the asphyxial changes. Glutamic and aspartic acid are present in the cortex and can elicit SD. These compounds thus fulfill the above requirements for a compound involved in the propagation of SD. The mechanism of propagation might be similar to that proposed by GRAFSTEIN (1956*a*), but instead of potassium ions, the stimulus initiating SD might release glutamic (aspartic) acid from the nerve cells and fibres which as in the case of the externally applied amino acids might produce the changes typical for SD in adjacent neurones which would release more of these compounds, etc. Both GRAFSTEIN's potassium hypotheses and the glutamic acid mechanism proposed above could explain the slow propagation of SD. Metrazol was shown in this paper to diminish the threshold for SD of glutamic and aspartic acid. It has been found previously

that Metrazol facilitates the initiation of spreading depression by neuronal stimulation (VAN HARREVELD and STAMM, 1955). This latter observation could be explained by the assumption made above that Metrazol inhibits the metabolic use of glutamic and aspartic acid. Glutamic and aspartic acid seem for this reason more likely compounds to be involved in the propagation of SD than potassium. If glutamic (aspartic) acid is involved in the mechanism of SD it is likely that it also plays a major part in the asphyxial cortical changes which have a striking resemblance to this phenomenon.

The drop in cortical conductivity during SD and after asphyxiation has been explained by an ion transport from the extra- into the intracellular compartment (VAN HARREVELD and OCHS, 1956, 1957). To maintain osmotic equilibrium this must be accompanied by a movement of water. The expected swelling of apical dendrites under these circumstances has been demonstrated (VAN HARREVELD, 1957, 1958). As a mechanism for the ion transport an increase in sodium permeability of the neuronal membrane was proposed which would create a Donnan situation in which extracellular sodium and chloride ions would be transported into the intracellular compartment. It was postulated above that the membrane changes resulting in the ionic transport are produced by the presence of glutamic (aspartic) acid in the extracellular fluid. This view is strongly supported by a recent paper by AMES (1958) who found in experiments with the retina of the rabbit *in vitro* that glutamate in relatively low concentrations (from 0·5 to 5 m-moles/l.) caused during the first 10 minutes of its application an increase of intracellular water, sodium and chloride. This effect was produced equally well with the right as with the left rotating stereoisomer.

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SUMMARY

Extracts of rabbit pallium cause contractions of crustacean muscles in relatively high dilutions. By adsorption on a Dowex 50 column and elution with dilute hydrochloric acid the extracts were purified. It was possible in this way to separate the active compound from the potassium ion present in the crude extracts. Chromatography and electrophoresis experiments suggested glutamic acid as the active compound. Indeed, L-glutamic acid caused contractions of crustacean muscles in a mean threshold concentration of 0·035 m-moles/l. (5 μg/ml). No other compound related with L-glutamic acid chemically or metabolically elicited muscle contractions in comparable dilutions.

Metrazol sensitizes the rabbit cortex for the initiation of SD by neuronal activity. It also sensitizes the cortex for the initiation of SD by brain extracts. The extracts purified by adsorption on a Dowex 50 column were very active in eliciting spreading depression. This suggested that the same compound is involved in the initiation of SD as in the contraction of crustacean muscle. Indeed it was found that L-glutamic acid initiates spreading depression when topically applied to the cortex in a mean concentration of 15 m-moles/l. Administration of Metrazol reduced this threshold concentration to 5·5 m-moles/l. About equally potent were L-aspartic acid and L-asparagine. Less effective were L-α-aminoadipic acid, L-glutamine, L-proline and succinic acid. Surprising was the great activity of D-glutamic acid which produced spreading depression in a mean threshold concentration of 3·6 m-moles/l. No effect of glutamic acid was found on the rabbit blood pressure, on the contractions of rabbit intestine, on the heart and blood vessels of the frog and on crustacean nerve.

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ALKALI-STABLE PHOSPHOLIPIDS DURING THE DEVELOPMENT OF THE RABBIT BRAIN*

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IN a previous study (EDGAR, 1957*a*) the amount of alkali-stable phospholipids (measured according to SCHMIDT *et al.*, 1946) in the rabbit brain during the first year of life was compared with the process of myelination judged from histological sections. As stated in detail elsewhere (EDGAR, 1957*b*) those observations have raised among others the problem that alkali-stable phospholipids occur in the unmyelinated telencephalon of newborn rabbits. This does not agree with the view suggested among others by JOHNSON, MCNABB and ROSSITER (1949) and BRANTE (1949) that this fraction is mainly localized in the myelin sheaths. Furthermore the level of alkali-stable phospholipids (expressed as a percentage of weight) was found to decrease after the age of 3–4 months, i.e. after the completion of histologically apparent myelination. This phenomenon has not been reported previously in the literature. In accordance with the initial report by SCHMIDT, BENOTTI, HERSHMANN and THANNHAUSER (1946) the alkali-stable phospholipid fraction is usually referred to as 'sphingomyelin'. BRANTE (1949) and DAWSON (1954) showed, however, that this fraction does not consist exclusively of sphingomyelin but contains a considerable amount of non-sphingomyelin phosphorus. Therefore the present study was undertaken to investigate to what extent true sphingomyelins are concerned with the observations aforementioned.

MATERIAL AND METHODS

The telencephalons of 1–3 days old, 3–4 months old and 1–2 years old rabbits of impure stock were studied. At these stages the main land-marks in the quantitative changes of the alkali-stable phospholipids were observed in our previous investigations.

Preparation and subsequent subdivision of the brain has been described in detail before (EDGAR, 1957*a*). With this technique the rostral quadrigeminal bodies are included in the samples designated as 'telencephalon'.

Pilot experiments indicated that the method of determining choline phospholipids should be preferably applied to extracts of dry tissue (instead of using wet tissue as in the earlier studies). Therefore the samples were first lyophilized and then extracted for 2 hr with hot chloroform-methanol (1 : 3 v/v).

In the extracts alkali-stable phospholipids and glycosphingosides were determined according to the method of SCHMIDT *et al.* (1946) and BRÜCKNER (1941) respectively, as described in detail elsewhere (EDGAR, 1956).

In accordance with Brante sphingomyelins were estimated as alkali-stable choline lipids, i.e., the difference between the total amount of choline-containing phospholipids (total choline lipids) and the amount of choline-containing phospholipids, which are hydrolysed by KOH in SCHMIDT's procedure (alkali-stable choline lipids). For the determination of the total amount of choline phospholipids, hydrolysis was performed with saturated aqueous $Ba(OH)_2$ for 23 hr in rubber stoppered tubes at 100°C. In the hydrolysates choline was determined with the periodide method of APPLETON,

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La Dur, Levy, Steele and Brodie (1953). Choline phospholipid values are calculated by multiplying the quantity of choline in lipid hydrolysates by 6·4.

Initially an attempt was made to determine choline by the reineckate method. However, in view of technical difficulties encountered and of the disputed specificity of the reineckate method, it was felt that the periodide method published by Appleton *et al.* (1953) might be preferable. This procedure had to be modified as described by Smits (1957) for reasons put forward in the same paper. The procedure finally adopted yielded reproducible results, especially when applicated to extracts of previously lyophilized nervous tissue. Extracts of wet tissue could be used but this involves more technical difficulties and the results are less reliable. The choline lipid values measured with this method proved to be of the same magnitude as the values obtained on the same sample with the reineckate method (Table 1). Therefore the values for choline lipids in the present paper are appropriate for comparison with values reported by others working with the reineckate method. Statistical calculations are based on the methods of Fisher (1948).

Table 1.—Choline values (expressed as a percentage of dry weight) for the same sample measured with the reineckate method and the periodide method respectively

(Number of determinations indicated in brackets)

	Total	In alkali-labile fraction	In alkali-stable fraction
Reineckate method	1·77 (4)	1·25 (5)	0·52
Periodide method	1·65 (9)	1·18 (9)	0·47

RESULTS

These are presented in Table 2.

Sphingomyelins (alkali-stable choline-lipids). Subtraction of the values for alkali-labile choline lipids from those for total choline lipids yielded positive figures in two out of the four pooled samples and in nine out of the fourteen single samples from 0–3 days old rabbits. The mean value for the latter group of observations differs significantly from zero ($P < 0{\cdot}01$). It is not possible to decide, however, whether this observation indicates the presence of a small quantity of sphingomyelin or reflects some small systematic error (e.g. adsorption of choline on to the filter paper resulting in low values for alkali-stable choline lipids). As expected the well-myelinated forebrain of the older rabbits contained much larger quantities of sphingomyelin. In contrast to the observations on the total alkali-stable phospholipid fraction, however, the choline containing part of this fraction (representing sphingomyelin) was found to increase significantly during the period between about 3 months age and 1 year ($P < 0{\cdot}02$)

Glycosphingosides. This fraction paralleled the sphingomyelin fraction. The increase of the glycosphingoside content between the age of 3 months and of 1 year was found to be significant ($P < 0{\cdot}01$).

Alkali-stable phospholipids. The mean value obtained for this fraction in the 0–3 days old animals differs significantly from zero ($P < 0{\cdot}01$). Subtraction of values for alkali-labile phospholipids from values for total phospholipids yielded positive figures in all samples studied.

Between the age of 3 months and of 1 year a decrease in the alkali-stable phospholipid content, although not significant ($0{\cdot}2 < P < 0{\cdot}3$), would appear to take place

in view of the fact that significance could be well established ($P < 0{\cdot}001$!) for similar observations made before (EDGAR, 1957*a*). The variance between the two studies in this regard could be attributed to the fact that the alkali-stable phospholipid content of a brain homogenate measured in lyophilized samples proved to be smaller than the one found in its wet samples. For the same reason the quantity of alkali-stable phospholipids other than sphingomyelin is not accurately measured by subtraction of sphingomyelin values from the values for alkali-stable phospholipids arrived at in the present study.

TABLE 2.—MEANS OF VALUES FOR VARIOUS LIPID FRACTIONS, EXPRESSED AS A PERCENTAGE OF DRY WEIGHT
(Standard deviations in brackets. N = number of samples)

Age	N	Phospholipids			Choline-containing lipids			Glyco-sphingo-sides
		Total	Alkali-labile	Alkali-stable	Total	Alkali-labile	Alkali-stable (sphingo-myelin)	
0–3 days	14*	20·5 (±2·3)	19·4 (±2·4)	1·02 (±0·55)	10·5 (±0·67)	10·1 (±0·77)	0·44 (±0·49)	1·84‡
	4†	18·8 (±2·4)	18·0 (±2·4)	0·8 (±0·35)	9·9 (±0·13)	9·7 (±0·48)	0·2 (±0·53)	1·60‡
3–4 months	15	24·3 (±1·8)	20·8 (±1·4)	3·5 (±0·93)	10·24 (±0·56)	8·58 (±0·42)	1·66 (±0·32)	7·54 (±0·55)
1–2 years	7	24·6 (±1·8)	21·6 (±1·3)	3·0 (±0·73)	10·1 (±0·70)	7·9 (±0·56)	2·2 (0·74)	8·9 (±0·48)

* Fourteen single animals.
† Four pooled litters.
‡ Mean of two observations.

DISCUSSION

In general the conclusions are that sphingomyelins are neither concerned with the presence of alkali-stable phospholipids in the telencephalon of newborn rabbits, nor with the puzzling decrease of the alkali-stable phospholipid values after the age of 3–4 months.

In the histologically unmyelinated samples of newborn animals, only traces of sphingomyelin at the most, were found. Its presence can be easily attributed to the fact that some of the animals were already 2 days old and that the samples contain a small part of the mesencephalon where myelination has already commenced in some tracts. The presence of a more considerable quantity of alkali-stable phospholipids in these samples is apparently due to non-sphingomyelin compounds and therefore does not contradict the assumption that sphingomyelin is localized in the myelin sheath. This view is supported by the fact that SCHUWIRTH (1940) could not find

TABLE 3.—COMPARISON OF PREVIOUSLY PUBLISHED RESULTS (EDGAR, 1957*a*) WITH THE RESULTS OF THE PRESENT STUDY RECALCULATED ON A WET WEIGHT BASIS FROM THE DATA PRESENTED IN TABLE 2
(figures in brackets indicate number of samples studied)

Age	0–3 days (data from first row of Table 2)	3–4 months	1–2 years
WET WEIGHT (g)			
Present study	1·42 ± 0·13 (14)	6·98 ± 0·25 (16)	7·27 ± 0·36 (6)
EDGAR (1957*a*)	1·51 ± 0·49 (18)	6·20 ± 0·49 (10)	8·36 ± 0·58 (10)
% DRY WEIGHT			
Present study	10·9 ± 0·57 (14)	19·1 ± 0·96 (15)	19·8 ± 1·0 (7)
(EDGAR, 1957*a*: not determined)			
ALKALI-STABLE PHOSPHOLIPIDS			
Present study	0·11 ± 0·06 (14)	0·66 ± 0·18 (15)	0·59 ± 0·15 (7)
EDGAR (1957*a*)	0·29 ± 0·25 (10)	1·48 ± 0·18 (10)	0·97 ± 0·17 (10)
TRUE SPHINGOMYELINS (Alkali-stable choline lipids)			
Present study	0·048 ± 0·053 (14)	0·32 ± 0·06 (15)	0·43 ± 0·14 (7)
(EDGAR, 1957*a*: not determined)			
GLYCOSPHINGOSIDES			
Present study	0·20 (4)	1·44 ± 0·11 (15)	1·76 ± 0·10 (7)
EDGAR (1957*a*)	0·21 ± 0·04 (11)	1·46 ± 0·09 (10)	1·75 ± 0·20 (10)

sphingomyelin in unmyelinated human nervous tissue using classical methods of isolation. The increase of sphingomyelin between the age of 3 months and 1 year shows that the decrease of the total alkali-stable phospholipid fraction during this period reflects a decrease of its non-sphingomyelin compounds and does not concern sphingomyelin.

It is of interest to compare the results of the present study with the observations reported on in a previous paper (EDGAR, 1957*a*). Since no dry weight determinations were made in the latter study this can only be done on a wet weight basis (Table 3).

The agreement between the glycosphingoside values obtained in the two studies is striking. The values for alkali-stable phospholipids observed in the present study, however, are much smaller than those reported in 1957 for corresponding age groups. Since in the present study extracts were prepared from lyophilized tissue instead of from wet tissue, we tried to test whether this technical difference could be the cause of the difference in phospholipid values. Determinations of alkali-stable phospholipids in wet and dry samples from the same brain homogenate and in a wet and a lyophilized hemisphere of the same brain, generally yielded lower values for the dried samples than for the wet ones. The reverse was found for the determination of choline phospholipids which yielded larger values in extracts from dried samples. No conspicuous differences, however, were found between the glycosphingoside values determined in wet and in dry samples respectively.

This is in agreement with the fact that the glycosphingoside values found in the present study are similar to those reported on previously (Table 3).

Probably the above effect of freeze-drying is accounted for in the values obtained at present for alkali-stable phospholipids. Consequently the differences between these values and the values for sphingomyelin cannot be used for measuring the amount of non-sphingomyelin compounds present in the alkali-stable fraction and, therefore, were not entered in our tables. On the other hand the present study confirms that only a part of this fraction consists of sphingomyelin and demands for a more detailed investigation of the non-sphingomyelin part. A key question is to what extent we are dealing with a true lipid, with a non-lipid contaminant or with both.

The occurrence of non-sphingomyelin phosphorus in the alkali-stable phospholipid fraction was noted for the first time by BRANTE (1949). He suggested that it was derived from a "sphingomyelin-like compound having, instead of choline, another reineckate non-precipitable, or no N-base". On the basis of fractionation experiments the new compound was provisionally included in the kephalin group and designated as 'kephalin B'.

After BRANTE's initial report only a few papers pertinent to this problem have been published.

From an abstract of a paper it appears that partition of tissue phospholipids is still under investigation in SCHMIDT's laboratory (SCHMIDT and SPENCER, 1957). Like BRANTE these authors suggest that the alkali-stable phospholipid fraction contains a phosphosphingolipide which does not contain choline. DAWSON (1954) confirmed the occurrence of substances other than sphingomyelin in the alkali-stable phospholipid fraction. Furthermore he found that a part of the non-sphingomyelin lipid phosphorus in this fraction is removed by initially precipitating the tissue lipids with trichloroacetic acid at room temperature. The other part of the non-sphingomyelin lipid phosphorus is suggested to be derived 'from a new phospholipid or from some known phospholipid present in modified or combined form'. Recently CARTER *et al.* (1958) have reported the occurrence of a new alkali-stable phospholipid in egg yolk. This substance consists mainly of a phosphorylethanolamine derivative of batyl alcohol and contains no sphingosine. The authors relate their findings to the above observations concerning non-sphingomyelin phosphorus present in the alkali-stable phospholipid fraction. This brief survey shows that it is not correct to use terms like sphingomyelin or phosphosphingosides for the alkali-stable phospholipid fraction. Its non-choline containing compounds can be provisionally referred to as 'non-sphingomyellin part of the alkali-stable phospholipid fraction' until more information is available concerning their chemical structure. In this connexion we should like to speak of a 'kephalin B' problem rather than of a 'kephalin B' fraction. A definite answer to this problem might explain also the effect of freeze-drying on the results of alkali-stable phospholipid determinations.

Of equal importance is the problem of the localization of the non-sphingomyelin part of the alkali-stable phospholipid fraction. The only available data are those of BRANTE (1949) who suggests that 'kephalin B' is predominant in white matter. Sphingomyelins are also predominant in white matter and therefore one of us (EDGAR, 1957*a*) has tentatively related the increase and subsequent decrease of the total alkali-stable phospholipid fraction during development to the process of myelination, disregarding the unsolved problems concerning the nature of its non-sphingomyelin part. Since the present study shows that the decrease in alkali-stable phospholipids at the end of development concerns exclusively this part of the fraction, it becomes essential to substantiate the presumption that it is mainly present in white matter.

Pertinent studies are in progress and will be given in a subsequent paper. On the basis of their results it will be decided whether or not the developmental changes in the non-sphingomyelin part of the alkali-stable phospholipid fraction, regardless of its nature reflect an aspect of the biological process of myelination. In conclusion we would like to draw attention to a side observation, resulting from the present study.

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From Table 2 it can be inferred that the difference between all alkali-stable phospholipids and the choline-containing alkali-stable phospholipid fraction increase continuously during the whole period of development studied. This difference represents the non-choline-containing alkali-stable phospholipids, or in conventional terms the kephalin fraction (excluding 'kephalin B') and the acetalphospholipids. This indicates that these fractions possibly participate in the process of myelination and therefore should be studied in more detail. BRANTE (1949) has already stated that the serine-containing kephalins contribute to the myelin sheath. According to KLENK (1952), KOREY (1956) and STAMMLER (1952) this is also true of the acetalphospholipids.

SUMMARY

In order to analyse further problems raised by previous studied on myelination, alkali-stable phospholipids, sphingomyelins and glycosphingosides were determined in lyophilized samples of the telencephalon of rabbits at the ages of 0–3 days, 3–4 months and 1–2 years respectively. In the 0–3 days old animals, at the most traces of sphingomyelin were found.

Sphingomyelins and glycosphingosides increase proportionally between the ages of 3–4 months and 1–2 years.

Apparently sphingomyelins are related neither to the presence of alkali-stable phospholipids in the unmyelinated telencephalon of newborns, nor with the decrease of the alkali-stable phospholipid content after the age of 3–4 months, observed before and in the present investigation. The problem raised by these observations are probably connected with the occurrence of non-sphingomyelin compounds in the alkali-stable phospholipid fraction.

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AMINO ACID AND PROTEIN METABOLISM OF THE BRAIN—IV

THE METABOLISM OF GLUTAMIC ACID

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INTRODUCTION

THE evidence for a significant role of glutamic acid in the metabolism of nervous tissue rests almost exclusively on results obtained with isolated tissue preparations and on the fact of the high concentration of glutamic acid in this tissue (for reviews see WAELSCH, 1952, 1957; STRECKER, 1957). Although changes of glutamic acid concentration in insulin hypoglycemia (DAWSON, 1950; HIMWICH and SULLIVAN, 1956) and the results of perfusion experiments (BOULANGER *et al.*, 1950) are highly suggestive of a rapid glutamic acid metabolism, the rate of the conversion of the dicarboxylic acid has not been studied. Only very recently data on the rate of decarboxylation of glutamic acid to γ-aminobutyric acid* *in vivo* have become available (ROBERTS *et al.*, 1958). In order to contribute to the understanding of the *in vivo* metabolism of glutamic acid in the brain, the penetration of ^{14}C-labelled glutamic acid into this organ as well as its conversion to glutamine, Gaba, and GSH in brain, liver, kidney and muscle was studied.

Previous work in this laboratory showed no significant increase of the concentration of glutamic acid in the brains of animals in short time experiments when the concentration of the amino acid in the blood of rats or mice was raised from thirty to fifty times. The glutamic acid concentration of liver and muscle was moderately increased while a large increase was found in the kidneys. On the other hand, when glutamine was administered, the amide concentration increased in all organs, an increase which was accompanied by an elevation of glutamic acid concentration in liver, kidney and blood. It appears, therefore, that glutamine penetrates into the organs, and especially into brain, at a higher rate than glutamic acid, and that glutamine is deamidated rapidly to glutamic acid (SCHWERIN, BESSMAN and WAELSCH, 1950). The possibility cannot be dismissed that the failure to observe a net increase of glutamic acid in brain after the administration of the amino acid is the result of rapid metabolism and removal of any additional amounts, although it should be remembered that both in liver and in kidney increases in glutamic acid could be demonstrated after the administration of either the acid or its amide.

Recently, in further studies of the amino acid and protein metabolism of the brain, it was found that after systemic administration of minute amounts of the ^{14}C

* The following abbreviations are used in this paper: glutathione, GSH; γ-aminobutyric acid, Gaba; trichloroacetic acid, TCA.

labelled amino acids, lysine (LAJTHA, FURST, GERSTEIN and WAELSCH, 1957) and leucine (LAJTHA, 1959) exchanged rapidly between the circulating blood and brain or liver. In spite of this rapid exchange, no net increase in the concentration of these compounds in the brain could be found, unless very large quantities of the amino acids were administered over long time periods (LAJTHA, 1958). In view of these results it appeared probable that a rapid exchange of glutamic acid between blood and brain may take place without a detectable net uptake by the organ. In the course of this investigation, the uptake of ^{14}C-labelled glutamic acid and of glutamine by brain and other organs from the circulating blood, as well as the rate of further metabolism of the dicarboxylic acid and its amide, was studied.

For the separation of glutamic acid, glutamine, Gaba and GSH, certain features of the recently developed method for the micro determination of these compounds were utilized (BERL and WAELSCH, 1958).

EXPERIMENTAL

Material. Glutamic acid ^{14}C uniformly labelled (11·3 mc/m-mole) was obtained from Nuclear Chicago Corp. and glutamine ^{14}C uniformly labelled (0·93 mc/m-mole) from Merck & Co. of Canada. Amberlite XE 59 was furnished by Rohm & Haas Co. (courtesy of Mr. R. PERCIVAL) and Dowex 50 × 4 − 400 mesh by the Dow Co.

Animal experiments and preparations of organ extracts. The animals used were adult (about 100 day old) C_3H mice and adult Sprague–Dawley rats. Glutamic acid and glutamine were injected via the tail vein (for amounts see legends of Tables 2 and 4). The animals were killed by decapitation at the end of the experimental time period; brain and liver were excised, blotted free of blood on filter paper, and frozen in dry ice. The blood was collected in heparinized centrifuge tubes and centrifuged for 3 min at 18,000 × **g**, and the plasma was frozen in dry ice. Four mice or two rats respectively were pooled for each experimental point. The frozen tissues were homogenized in 5% trichloroacetic acid (TCA) and centrifuged. This procedure was repeated two more times and the extracts combined.

Principle of the separation method. The tissue extracts were first chromatographed on Amberlite XE 59 columns. This resin retained the TCA and permitted the separation of glutamine and Gaba in one fraction and of glutamic acid (free from aspartic acid and GSH) in another fraction. The glutamine and Gaba were then separated on Dowex 50 and finally both the glutamic acid and the glutamine (the latter after hydrolysis to glutamic acid) were further purified on alumina columns. In those experiments in which GSH was also determined, the glutamic acid and GSH were chromatographed on alumina columns. The methods of separation and preparation of the Dowex and alumina columns were essentially those described by BERL and WAELSCH (1958).

Separation of the amino acids. Amberlite XE 59 was converted to the acetate form by stirring the resin for 2 hr with 10 vols 4N-acetic acid, filtering on a Buchner funnel, and washing with 10 vols 2N-acetic acid. This procedure of stirring and filtering was repeated three times with 2N-acetic acid, twice with water, and then three times with 5 vols 0·2 M-ammonium acetate buffer pH 5. The resin was stored in the pH 5 buffer, and, before use, was again washed on a Buchner funnel with the pH 5 ammonium acetate buffer. The resin must be washed just before use, since otherwise it colours the eluate. Finally it was poured into glass columns of 11 mm i.d. with an end tapered down to 2 mm i.d. and closed with a plug of glass wool. For 20 ml TCA extract, a 180 mm long resin column was used. The extract was put through the column and the column was washed with subsequent portions of 0·5 mole acetic acid. TCA was retained on the Amberlite column. Glutamine and Gaba came through in the first 53 ml of eluate (20 ml original extract + 33 ml acetic acid wash), while glutamic acid was eluted in the following 68-108 ml fraction.

The 53 ml eluates from the Amberlite columns were concentrated to 10 ml by lyophilization, the pH was adjusted to 2, and glutamine and Gaba were separated out on Dowex 50 × 4 − 400 mesh, as described by BERL and WAELSCH (1958); 5 ml fractions were placed on the columns and the eluates combined for determination and counting.

The Gaba fraction showed one spot on paper (phenol, water). After the determination of the

specific activity it was twice recrystallized from aqueous ethanol after addition of unlabelled Gaba with no change in specific activity. Glutamine was hydrolysed to glutamic acid with 2N-HCl at 100° for 2 hr. The HCl was then removed *in vacuo*. Both the hydrolysed glutamine and glutamic acid were further purified by adsorption and elution on alumina columns. Aliquots (5 ml) of the neutralized glutamic acid solutions were passed through 4 × 0·5 cm columns. The columns were washed twice with 2 ml of H_2O and the glutamic acid was eluted from the columns with 5 ml of 0·1 N-NH_4OH. Aliquots of the NH_4OH eluates were used for counting and for the determination of glutamic acid.

Determination of specific activity. For the determination of glutamic acid, glutamine, Gaba and GSH the method of BERL and WAELSCH (1958) was used. For the determination of radioactivity the solutions were plated on stainless steel planchets (1·25 in. diameter) and counted in a Nuclear Chicago C 110 B gas flow counter. Correction was made for self-absorption.

Efficiency of the methods of separating the various compounds. The degree of separation may be seen from the results of one representative experiment where ^{14}C glutamic acid is added to a TCA filtrate of brain corresponding to 1 g of tissue per 20 ml of 5% TCA (Table 1).

TABLE 1.—DISTRIBUTION OF COUNTS AFTER ADDITION OF ^{14}C GLUTAMIC ACID TO TCA EXTRACT OF BRAIN TISSUE

Step	Sample	Total counts	Glutamic acid (%)
1	Original solution*	101,000	100
2	Eluted from Amberlite, glutamine fraction	1940	2
3	Eluted from Amberlite, intermediate fraction	98	0·1
4	Eluted from Amberlite, glutamic acid fraction	91,400	91
5	Eluted from Amberlite, post glutamic acid fraction	7540	7·5
6	Sample 2 hydrolysed and purified on alumina	580	0·6
7	Sample 2 twice purified†	27	0·03
8	Gaba fraction purified on Dowex	18	0·02

* 101,000 counts ^{14}C glutamic acid were added to 20 ml of TCA extract of 1 g brain tissue.

† To the glutamine fraction as eluted from the Amberlite column ^{12}C glutamic acid was added and passed through alumina to remove the last traces of glutamic acid. The glutamine was subsequently hydrolysed and purified by alumina adsorption as described in the text.

Routinely, separations were carried through steps 1 to 6 and 8. Step 7 was used when a very high degree of separation was desired. It is apparent that when necessary the various fractions may be further purified by repetition of some of the steps described above. For the purpose of our study the separation of the different compounds was sufficient and the error introduced by impurities from other fractions was always below 2 per cent.

RESULTS

The concentrations of glutamic acid and related compounds in rat and mouse tissues found in the present study are in agreement with the values found in previous studies (SCHWERIN *et al.*, 1950; WAELSCH, 1951, 1957; BERL and WAELSCH, 1958).

The distribution of radioactivity in rat organs after the administration of uniformly labelled ^{14}C glutamic acid is shown in Table 2. Five minutes after administration, plasma glutamic acid has the highest specific activity but the glutamic acid in the organs is also significantly labelled. 'Total' radioactivity (Table 3) is much higher in the organs than in the plasma, a result which shows that most of the plasma glutamic acid had exchanged with glutamic acid of the tissue and of the liver particularly, which contains the higher 'total' amount of radioactivity. The brain glutamic acid is also significantly radioactive. In all organs, the specific activity of the glutamic acid

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TABLE 2.—DISTRIBUTION OF RADIOACTIVITY IN RAT ORGANS 5 MIN AFTER ADMINISTRATION OF ^{14}C GLUTAMIC ACID

	Experiment 1			Experiment 2		
Organs	Glutamic acid	Glutamine	GSH	Glutamic acid	Glutamine	GSH
Plasma				3200	670	
Brain	32	27	18	43	17	16
Liver	800	530	110	1800	460	130
Kidney				860	470	560
Muscle				400	46	45

Two rats in each experiment received i.v. (tail vein) 0·1 ml of a solution containing 5 μc/ml uniformly labelled ^{14}C glutamic acid. Each animal received 11 γ glutamic acid comprising 7 per cent of the glutamic acid circulating in its plasma. All values are corrected for counts introduced by blood remaining in the excised organ (LAJTHA *et al.*, 1957). Values expressed as counts/minute per μmole.

TABLE. 3.—TOTAL RADIOACTIVITY IN WHOLE RAT ORGANS*

	Experiment 1			Experiment 2		
	Glutamic acid	Glutamine	GSH	Glutamic acid	Glutamine	GSH
Plasma†				6400	4700	
Brain	640	220	81	860	140	72
Liver	18,000	20,000	6900	40,000	17,000	8200
Kidney				14,000	1600	4000
Muscle†				30,000	12,000	4700

* Derived from Table 2; expressed as counts/min in the whole organ.
† Estimated total weight.

fraction is higher than that of glutamine, an indication that the bulk of glutamic acid entered the organs as the acid. Because the amount of glutamic acid administered was small, we are dealing most probably with exchange but not with a net uptake.

Table 4 summarizes the results of similar experiments on mice. Uniformly labelled ^{14}C glutamic acid was injected and after the experimental period the animal was sacrificed and the concentration and radioactivity of the compounds determined. The results confirm in their essential aspects those obtained in the experiments with rats. In short term experiments (2–10 min) plasma glutamic acid has usually the highest specific activity although liver glutamic acid sometimes exceeds it. Glutamic acid of other organs is also significantly labelled, a finding which demonstrates a relatively rapid exchange of plasma glutamic acid with that of tissues. In brain and liver, the specific activities of glutamic acid and glutamine are close, glutamine occasionally having a higher specific activity. In Gaba of brain and in GSH of liver and brain a significant number of counts are found. Again, quantitatively, it can be seen that most of the radioactivity disappeared from the plasma and is widely distributed

throughout the organism as glutamic acid and its metabolic products. It should be noted that in many of the short time experiments the specific activity of blood plasma glutamine exceeded that of brain or liver glutamic acid or glutamine.

It is apparent that the animal rapidly converts glutamic acid to glutamine with a

TABLE 4.—SPECIFIC ACTIVITY OF COMPOUNDS AFTER INJECTION OF ^{14}C GLUTAMIC ACID

Duration of experiment (min)	Organ	Specific activity of compounds (counts/min per μmole)			
		Glutamic acid	Glutamine	γ-Amino-butyric acid	Glutathione
	Plasma	45,000	5200		
2	Brain	85	57	31	25
	Liver	2800	2100		150
	Plasma	12,000	1900		
	Brain	15	13	3·4	8·3
2	Liver	1100	510		38
	Red Blood Cells	1900	220		65
	Kidney	704	390		340
	Plasma	8300	4500		
3	Brain	620	160	20	
	Liver	12,000	20,000		
	Plasma	6000	1900		
5	Brain	90	36	33	
	Liver	1200	850		
	Plasma	22,000	4100		
5	Brain	95	78	34	51
	Liver	2400	2000		200
	Plasma	8000	3000		
10	Brain	920	230	60	
	Liver	9200	21,000		

Four adult mice were pooled for each point. They were injected with ^{14}C glutamic acid intravenously. 0·5 μc and 7 γ glutamic acid was injected in each animal corresponding to about 30 per cent of the plasma glutamic acid content and 4 per cent of blood volume. All values were corrected for counts introduced by blood remaining in the excised organ.

resultant rapid rise in specific activity of plasma glutamine. It was thought that the situation may be clarified if the specific activity of glutamine could be diluted by the injection of unlabelled glutamine along with the radioactive glutamic acid, and vice versa.

The results of such experiments are presented in Table 5. For the first two experiments ^{14}C glutamic acid was injected into mice together with ^{12}C glutamine. In experiment 1, relatively large amounts of ^{12}C glutamine (approximately twenty times

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TABLE 5.—SPECIFIC ACTIVITIES OF GLUTAMIC ACID AND GLUTAMINE AFTER THEIR INJECTION INTO MICE

Exp.*	Compound injected	Duration of experiment (min)	Organ	Specific activity of compounds (counts/min per μmole)	
				Glutamic acid	Glutamine
1	^{14}C glutamic acid ^{12}C glutamine added	5	Plasma Brain Liver	20,000 170 3200	12,000 220 3500
2	^{14}C glutamic acid ^{12}C glutamine added	3	Plasma Brain Liver	130,000 77 1600	700 40 2000
3	^{14}C glutamine	5	Plasma Brain Liver	2500 69 1800	5000 200 3700
4	^{14}C glutamine ^{12}C glutamic acid added	5	Plasma Brain Liver	8300 97 3000	11,000 380 5900

* Four animals were used in each experiment. The following, contained in 0·2 ml, was injected into each animal.
(1) 2 mg ^{12}C glutamine + 7·5 γ ^{14}C glutamic acid, 400,000 counts/min. Injected within 2 sec.
(2) 4 mg ^{12}C glutamine + 7·5 γ ^{14}C glutamic acid, 400,000 counts/min. Injected slowly over a period of 3 min.
(3) 70 γ ^{14}C glutamine, 270,000 counts/min. Injected within 2 sec.
(4) 1 mg ^{12}C glutamic acid + 70 γ ^{14}C glutamine, 270,000 counts/min. Injected within 2 sec.

that normally present in the blood) had no effect on the specific activities of these two compounds in the liver and plasma. In experiment 2, with even larger amounts of ^{12}C glutamine (approximately forty times that normally present in the blood) dilution of the specific activity of the plasma glutamine was achieved when it was administered throughout the experimental period.

In the third experiment ^{14}C glutamine alone, and in the fourth experiment, ^{14}C glutamine together with ^{12}C glutamic acid was administered. In these experiments the glutamine was of higher specific activity than the glutamic acid in the plasma and liver, and this was also true for the brain.

These experiments again indicate the tremendous ability of the liver and the brain to quickly interconvert glutamic acid and its amide. When the specific activity of the plasma glutamic acid was considerably higher than the specific activity of the plasma glutamine, this was reflected in their relative specific activities in the brain. When the specific activities of the plasma glutamic acid and glutamine approached equality this also became true for the brain. This again supports the probability that plasma glutamic acid exchanges with brain glutamic acid. Glutamic acid and glutamine may have different rates of entry into the central nervous system but this is difficult to evaluate because five to ten times as much glutamine as glutamic acid

passes through the brain in the plasma in a given period of time. Therefore, any elevation of the brain glutamic acid specific activity over that of the brain glutamine specific activity becomes that much more significant.

Table 6 contains the results of experiments of longer duration. After several hours the specific activities of the compounds decreased in the organs.

TABLE 6.—DISTRIBUTION OF RADIOACTIVITY *in vivo* AFTER LONGER EXPERIMENTAL PERIODS

Duration of experiment (hr)*	Organ	Specific activities (counts/min per μmole)		
		Glutamic acid	Glutamine	γ-Aminobutyric acid
2	Plasma	1600	400	36
	Brain	61	47	
	Liver	92	100	
5	Plasma	2000	890	10
	Brain	12	21	
	Liver	34	92	
19	Plasma	2000	1300	(2)
	Brain	20	4	
	Liver	22	80	

* Each time point is the average of two experiments. For experimental conditions see legends for Table 4.

Table 7 contains the results of *in vitro* experiments. As shown, brain tissue is capable of producing glutamine and Gaba from glutamic acid. Liver also is capable of amide formation, but in these unfortified homogenates the speed of glutamic acid metabolism is much slower than in the *in vivo* experiments.

TABLE 7.—CHANGES IN SPECIFIC ACTIVITIES *in vitro*

Organ	Time of experiment (min)	Specific activity in the fractions (counts/μmole)		
		Glutamic acid	Glutamine	γ-Aminobutyric acid
Brain	0	13,000	0	0
	15	11,000	400	800
Liver	0	18,000	0	
	15	12,000	100	

Whole homogenate in 0·9% saline was incubated at 37° for 15 min. Brain was a 1 : 4 homogenate. Liver was a 1 : 2 homogenate.

DISCUSSION

The pivotal position of glutamic acid and its keto acid in amino acid and carbohydrate metabolism suggests their rapid metabolism, a fact which is indicated by many *in vitro* studies. The manifold metabolic functions of glutamic acid and the synthesis and oxidation of ketoglutaric acid through the citric acid cycle present obvious difficulties when an accurate rate of utilization and a preference for certain metabolic pathways are to be ascertained. When labelled with ^{14}C the marker will be rapidly diluted and distributed along the various metabolic pathways. The data presented in this report give an indication of the rapidity with which the system glutamic acid-glutamine operates *in vivo* in liver, kidney and brain, and of the rate at which glutamic acid is converted into Gaba in the central nervous system.

It should be noted that in some short time experiments the specific activity of the plasma glutamine exceeded not only that of the liver and kidney glutamine but also that of liver and kidney glutamic acid. Such a finding could be explained by an admixture of glutamic acid of high specific activity with the glutamine. Owing to the five to ten times higher concentration of glutamine than glutamic acid in plasma, the admixture of glutamic acid with the glutamine would have to be considerable even with a high specific activity of the former. Direct tests of the ability of the method to separate glutamic acid and glutamine indicate that the mutual impurities are not above the 1 per cent level (Table 1). Therefore, it has to be concluded that the source of the plasma glutamine is an organ other than those analysed—brain, liver, kidney—or that the highly labelled glutamic acid is converted into glutamine, e.g. without the acid or amide being mixed within the organ with the respective compounds. Part of glutamine would then be returned undiluted to the plasma and part would mix with the organ glutamine over a longer period. Glutamine of higher specific activity than the organ glutamic acid or glutamine may result from its synthesis from the administered glutamic acid in the wall of the blood vessels or in a particular cell species of brain and liver, with a direct return to the circulating blood without dilution with the glutamic acid and glutamine of the respective organ. It is undoubtedly too early to speculate as to the place of glutamine synthesis but is should be remembered that glutamine synthetase occurs mainly in the microsomal preparations of the liver and brain (WAELSCH, 1958*a*; SACHS and NEIDLE, 1956). It could be visualized that the endoplasmic reticulum is the compartment in which glutamine is synthesized without being mixed with the amide occurring in other compartments of the cell, part of the newly synthesized glutamine being returned to the plasma without extensive dilution. On the basis of this reasoning, metabolic products of substances metabolized by the endoplasmic reticulum of an organ may reappear in the blood with a specific activity higher than found with the compound isolated from the whole organ. The assumption that glutamine is synthesized in a compartment and in a short time interval does not mix with the organ glutamine finds support in the observation that administration of unlabelled glutamine in addition to the ^{14}C-labelled glutamic acid did not dilute the specific activity of the plasma glutamine (Table 5, experiment 1). It appears therefore that the administered glutamine did not mix with the amide formed from the administered glutamic acid. It is not possible to decide at the present time whether these results indicate different pathways of removal and compartments of metabolism of glutamine and glutamic acid in the same tissue or metabolism of different organs. Only short time experiments of a length which does not permit mixing of all the pools

in an organ can give a true picture of the complexity of the system as well as of the compartmentalization of pools of metabolites.

Irrespective of all such considerations, the conversion of glutamic acid into glutamine is undoubtedly extremely rapid. In some of the experiments, within 2 minutes the specific activity of the liver glutamine approached that of the liver glutamic acid. In others, the liver glutamine had actually a higher specific activity than the liver glutamic acid, a fact which indicates that non-labelled glutamic acid was synthesized rapidly while the glutamine was not diluted to such an extent. It can be seen that whenever ^{14}C-glutamic acid was administered, the specific activity of the tissue glutamic acid was higher than that of glutamine and vice versa. This finding indicates that glutamic acid as well as glutamine is largely taken up as such from the circulating blood. This finding is in agreement with the observation that also from the gut glutamic acid and glutamine are taken up into the portal vein without extensive interconversion (BESSMAN, MAGNES, SCHWERIN and WAELSCH, 1948).

As far as the brain is concerned, the experiments demonstrate that glutamic acid from the blood exchanges rapidly with that from the brain although no net uptake of glutamic acid by the brain can be demonstrated after excessive elevation of the blood glutamic acid. This finding is in keeping with the observation made previously with lysine (LAJTHA, 1958) and leucine (LAJTHA, 1959). Both amino acids exchanged rapidly with the pool of free amino acids of the brain without a noticeable net uptake when the blood concentration was elevated and the experimental period was not longer than 30 minutes. Because of the high metabolic activity of glutamic acid the failure to detect a net uptake by the brain may be due to the rapid metabolic removal of the dicarboxylic acid, but as pointed out above, it appears more likely that the blood–brain barrier does not permit a net uptake of the dicarboxylic acid although permitting a rapid exchange.

The specific activity of the brain glutamine was lower than that of the brain glutamic acid, a fact which suggests that the amide is derived from the acid in the organ itself, particularly in view of the potent glutamine synthetase occurring in brain (LAJTHA, MELA and WAELSCH, 1953). Experiments, to be reported in detail later, with intracerebrally injected glutamic acid have shown that brain rapidly converts this acid to glutamine. The lack of dilution of brain glutamine specific activity by intravenously administered ^{12}C glutamine when the ^{14}C was injected in the glutamic acid form suggests synthesis of ^{14}C glutamine from ^{14}C glutamic acid in the brain under our experimental conditions. However, since glutamine can enter the brain from the circulating blood, part of the brain glutamine may have its origin in plasma glutamine.

A calculation of the flux of glutamic acid from the plasma into the organs and particularly into the brain is made difficult not only by the fact that we are measuring on the decreasing activity time curve, where small difference in time expresses itself in large variation in the plasma concentration, but also by the fact that the true specific activity of the brain glutamic acid is difficult to ascertain because of its high metabolic activity. Such a calculation, which cannot be anything else than highly approximate (Table 8), gives values of a flux of 0·15–4·0 γ amino acid/g of brain per minute. These values are in the range of those found for leucine and lysine and suggest that the exchange rate for the tested amino acids irrespective of whether they are acidic (glutamic acid) or basic (lysine) is of the same order of magnitude.

While there may be some doubt as to the origin of brain glutamine it is very likely

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that the brain Gaba is derived from brain glutamic acid by decarboxylation. As can be seen from the respective specific activities, the rate of this conversion is quite rapid and suggests not only fast formation but also removal of Gaba. This finding is in full agreement with the data presented recently by ROBERTS *et al.* and does not suggest a metabolic inertness of Gaba, as may be surmised from its relative stability when incubated with brain cortex slices (ELLIOTT, 1958). It can also be seen from experiments reported in this paper that the conversion of ^{14}C glutamic acid into glutamine or Gaba is considerably slower in organ homogenates than *in vivo*.

TABLE 8.—TURNOVER OF FREE AMINO ACIDS IN BRAIN

		Flux	Half-life (min)	γ Substance/g fresh tissue	
				Plasma	Brain
Lysine	young	1·1–1·5	21–29	49	51
	adult	0·7–5·2	5–43	50	38
Leucine	adult	0·1–3·0	3–67	17	9·6
Glutamic acid	adult	0·15–4·0	200–5000	15	1600

Flux = γ amino acid/g fresh tissue per min exchanged.

Experiments of long duration support the evidence for the rapid metabolism of glutamic acid and its metabolic derivatives. Because of the speed of glutamic acid entry into the organs and its further metabolism, no definite conclusions can be drawn from these long term experiments, but the further breakdown of the acid and Gaba is shown. Plasma specific activities remain surprisingly high and indicate the possibility of a return of labelled compound from the organs to the blood and/or their binding to plasma proteins.

The data show the high metabolic activity of GSH, since considerable counts were found in GSH. Although this finding is in agreement with previous observations as to the high rate of GSH metabolism (WAELSCH and RITTENBERG, 1941) any definite statement as to this rate cannot be made without a knowledge of the distribution of counts in the amino acids. Although at the time of the original observation the turnover of the tripeptide seemed very rapid, it does not measure up to the rapidity of glutamic acid and glutamine conversion reported here and is probably in the range of the turnover of rapidly metabolized protein fractions of the various organs (cf. LAJTHA *et al.*, 1957*b*, WAELSCH, 1958*b*).

SUMMARY

The metabolism of ^{14}C-labelled glutamic acid was studied in intact rats and mice. It appears that the bulk of the administered amino acid enters brain, liver, kidney and muscle as such and not with prior conversion to the amide. The entry of the ^{14}C glutamic acid into the various organs was very rapid and within minutes the glutamic acid, glutamine, GSH fraction of the brain, liver and kidney, and the γ-aminobutyric

acid fraction of brain were significantly labelled. Within 2 minutes after the administration of ^{14}C glutamic acid the specific activity of the organ glutamine approached that of the dicarboxylic acid, a fact which indicates the rapidity of intraconversion of the glutamic acid–glutamine system.

In experiments of longer duration the specific activities of glutamic acid, glutamine, GSH, and γ-aminobutyric acid decreased as would be expected from their rapid metabolism. In a number of short time experiments the specific activity of plasma glutamine was higher than that of liver glutamic acid and glutamine. This finding is interpreted as suggesting that the amide is synthesized in a compartment of the organ (endoplasmic reticulum?) from labelled glutamic acid not diluted extensively by organ glutamic acid.

The flux of glutamic acid from the circulating blood into brain and liver is estimated. A method is presented for the efficient separation in isotope experiments of glutamic acid and its metabolic derivatives.

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CHEMICAL STUDIES OF PERIPHERAL NERVE DURING WALLERIAN DEGENERATION—X

IN VITRO INCORPORATION OF RADIOACTIVE INORGANIC PHOSPHATE INTO PHOSPHATIDES AND ACID-SOLUBLE PHOSPHORUS COMPOUNDS

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BODIAN and DZIEWIATKOWSKI (1950) found that when inorganic ^{32}P was administered to monkeys, some of the label was incorporated into the phospholipid of the peripheral nerves. SAMUELS, BOYARSKY, GERARD, LIBET and BRUST (1951) made similar observations for guinea pigs. Both groups of workers found that the specific radioactivity of the phospholipid in the distal portion of nerves degenerating after section was greater than that of the phospholipid in the intact nerves.

FRIES, SCHACHNER and CHAIKOFF (1942), using dog nerve, were the first to demonstrate that the phospholipid of a peripheral nerve could be labelled *in vitro* from inorganic ^{32}P. MAGEE and ROSSITER (1954) confirmed this finding with metabolizing fragments of cat sciatic nerve. They further demonstrated that when fragments of nerve degenerating after previous section were incubated under similar conditions, there was an increase in the incoporation of inorganic ^{32}P into the phospholipid. There was also an increase in the *in vitro* labelling of phospholipid in nerves removed from the animal at a time when they were regenerating after a crushing injury. More recently, ROSSITER, PRITCHARD and STRICKLAND (1958) reported that the *in vitro* incorporation of other isotopically labelled lipid precursors, such as, glycerol-1-^{14}C, choline-1:2-^{14}C, ethanolamine-1:2-^{14}C and serine-3-^{14}C, was increased in cat sciatic nerve degenerating after previous section (see also PRITCHARD and ROSSITER, 1958). MAJNO and KARNOVSKY (1958*a*) and KLINE, MAGEE, PRITCHARD and ROSSITER (1958) reported similar findings for the incorporation of acetate-1-^{14}C into the lipids of degenerating peripheral nerve.

It was suggested previously (ROSSITER *et al.*, 1958; KLINE *et al.*, 1958) that the increase in the incorporation of labelled precursors into the phospholipid of degenerating nerve may be associated with the well-known increase in the number of Schwann cells (ABERCROMBIE and JOHNSON, 1946). Since the work of GEREN (1954) has shown that the myelin sheath of a peripheral nerve is formed by the Schwann cells, the increase in the lipid labelling that occurs under conditions where the number of Schwann cells is increased, may represent an increase in the capability of the nerve to form myelin lipid, should a suitable opportunity occur. It was of interest, therefore, to determine whether all of the phosphatides of a nerve participated in the increase in the incorporation of inorganic ^{32}P, or whether the increase was confined to those phosphatides thought to be components of the myelin sheath. The technique of DAWSON (1954)

was used to determine the relative incorporation of inorganic ^{32}P into certain of the nerve phosphatides.

Inasmuch as the *in vitro* incorporation of inorganic ^{32}P into glycerophosphatides of cell-free preparations of liver and brain is known to depend upon both the concentration and the specific radioactivity of adenosine triphosphate (KENNEDY, 1953; MCMURRAY, STRICKLAND, BERRY and ROSSITER, 1957), it also was of interest to study the effect of nerve section on the concentration and ^{32}P-labelling of adenosine triphosphate and certain other acid-soluble phosphorus compounds in peripheral nerve.

METHODS

Cat sciatic nerves. The right sciatic nerve of a number of cats was sectioned at the level of the greater trochanter of the femur. Details of the operation have been described previously (JOHNSON, MCNABB and ROSSITER, 1949). No attempt was made to control the age, sex or weight of the animals, since the opposite side was left intact to serve as a control. After 8, 16 or 32 days the animals were killed and the segment of nerve distal to the site of operation was removed, together with a similar length of intact left sciatic nerve. Each nerve was cleaned of adherent fatty and connective tissue; the epineurium was stripped off and the nerve was rapidly weighed. It was cut into small lengths (about 1 mm) and transferred to a 20 ml beaker to which had been added 2·9 ml Krebs–Ringer medium containing 0·01 M-glucose and buffered at pH 7·4 with 0·05 M-2-amino-2-hydroxymethyl-propane-1:3-diol (tris). After the addition of 0·1 ml neutralized carrier-free inorganic ^{32}P, supplied in the form of $H_3{}^{32}PO_4$ by the Atomic Energy of Canada Limited, the beaker and contents were incubated at 37·5° under continuous flow of oxygen in a Dubnoff shaking unit (Precision Scientific Instrument Co., Chicago). In the experiments in which the hydrolysis products of the labelled lipid were to be isolated chromatographically, 500 μC inorganic ^{32}P was added to each beaker and the samples were incubated for 2 hr. In the experiments in which the acid-soluble phosphates were to be separated, 100 μC inorganic ^{32}P was added to each beaker and the samples were incubated for 90 min.

After incubation the nerve fragments were transferred to a mortar and the excess liquid was poured off. The tissue was then frozen with liquid nitrogen and ground to a fine powder. In the lipid experiments the removal of contaminating acid-soluble P and the extraction of the lipids were carried out as described by FINDLAY, MAGEE and ROSSITER (1954). In the experiments where the acid-soluble phosphates were to be isolated, the nerve powder was dispersed in 2 ml of ice-cold 20% (w/v) trichloroacetic acid (TCA) with the aid of a Potter–Elvehjem homogenizer. The dispersion was centrifuged in the cold and the supernatant fluid was made up to a volume of 2 ml.

Rat sciatic nerves. The right sciatic nerve of a number of rats was sectioned high in the thigh. After 12 days the nerves were removed. Details of the operation and the preparation of the nerves for analysis were described by MANNELL (1952). After the nerves had been weighed, they were frozen in liquid nitrogen, ground to a fine powder, and dispersed in 1 ml of ice-cold 20% TCA with the aid of a Potter–Elvehjem homogenizer. The dispersion was centrifuged in the cold and the supernatant fluid was made up to a volume of 1 ml.

Chromatographic procedures. In the lipid experiments, a portion of the lipid extract containing approximately 275 μg lipid P was taken to dryness under reduced pressure at 40°. The residue was extracted with chloroform: methanol (1 : 1, v/v) and the extract was carried through the mild alkaline-hydrolysis procedure of DAWSON (1954). The water-soluble hydrolysis products were purified and subjected to two-dimensional paper chromatography with the phenol: aq. NH_3 soln. and *tert.*-butanol: TCA solvents described by DAWSON (1954). By this method glycerylphosphorylcholine (GPC), glycerylphosphorylethanolamine (GPE), glycerylphosphorylserine (GPS), glycerophosphate (GP) and a hydrolysis product of diphosphoinositide (IP) were separated. The GPC, GPE and GPS are derived from phosphatidyl choline, phosphatidyl ethanolamine and phosphatidyl serine respectively. According to DAWSON (1954), the GP is derived from phosphatidic acid, but it is possible that some GP arises from other lipids. The spots were located, identified, eluted with water, ashed and the specific radioactivity determined as described by MCMURRAY *et al.* (1957).

In the acid-soluble P experiments, 500 μl of the appropriate TCA extracts were applied to washed Whatman No. 4 filter paper and phosphorus compounds were separated by the procedure of KREBS

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and HEMS (1953), as described by BERRY and MCMURRAY (1957). In this way it was possible to determine the concentration and the specific radioactivity of inorganic P, adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine-5′-monophosphate (AMP).

RESULTS

The specific radioactivities of the hydrolysis products of the individual phosphatides of cat sciatic nerve fragments are compared with similar findings for guinea pig brain slices in Table 1. The brain slices (100 mg fresh tissue) were incubated in the presence of 50 μc inorganic ^{32}P, whereas the nerve fragments (approximately 500 mg fresh tissue) were incubated in the presence of 500 μc inorganic ^{32}P. In each instance the concentration of inorganic P in the medium was the same. The mean specific radioactivity of the total lipid P for the brain and nerve preparation was found to be 110·9 and 98·2 counts/min per μg P, respectively. Although these figures are not strictly comparable, it is evident that, for the incorporation of inorganic ^{32}P into phospholipid, guinea pig brain is much more active than cat peripheral nerve. It is of interest that MAJNO and KARNOVSKY (1958*b*) recently reported that rat brain was much more active in this regard than rat sciatic nerve.

TABLE 1.—SPECIFIC RADIOACTIVITY OF THE HYDROLYSIS PRODUCTS OF THE INDIVIDUAL PHOSPHATIDES OF CAT SCIATIC NERVE FRAGMENTS AND GUINEA PIG BRAIN SLICES

Tissues incubated for 2 hr at 37·5° in a Krebs–Ringer medium buffered at pH 7·4 with tris buffer containing glucose and inorganic ^{32}P. Total volume, 3 ml; gas phase, O_2; specific radioactivities are relative to total lipid P = 100.

	Cat sciatic nerve fragments mean ± S.E.M.	Guinea pig brain slices mean ± S.E.M.
Total lipid P	100	100
GPC	215 ± 18 (8)*	101 ± 16 (8)*
GPE	13 ± 1 (8)	12 ± 1 (8)
GPS	8 ± 2 (8)	11 ± 1 (8)
GP	1890 ± 320 (8)	1690 ± 270 (7)
IP	1060 ± 390 (8)	1560 ± 100 (7)

* The figure in parentheses equals the number of mean experimental values used in the determination.

In Table 1 the results are expressed relative to a value of 100 for the specific activity of the total lipid P. Whereas the specific radioactivity of the GPC in guinea pig brain slices approximated to that of the total lipid P, the specific radioactivity of the GPC in cat sciatic nerve fragments was more than twice that of the total lipid P.

With both brain slices and nerve fragments the GPE and GPS were poorly labelled and the GP and IP were strongly labelled relative to the specific radioactivity of the total lipid P.

Table 2 shows that the specific radioactivity of the total lipid P of nerves studied 16 days after section was approximately double that for intact nerves from the same

TABLE 2.—SPECIFIC RADIOACTIVITIES OF THE HYDROLYSIS PRODUCTS OF THE INDIVIDUAL PHOSPHATIDES OF INTACT AND DEGENERATING (16 DAYS AFTER SECTION) SCIATIC NERVE OF THE CAT

Specific radioactivity expressed as counts/min per μg P

		Expt. 1	Expt. 2	Expt. 3	Mean % of intact nerve
Total lipid P	Intact control	94·4	97·6	86·7	
	Degenerating	212·0	194·5	134·8	194
GPC	Intact control	207	300	225	
	Degenerating	706	818	476	273
GPE	Intact control	9·4	15·4	14·3	
	Degenerating	20·3	33·4	22·8	196
GPS	Intact control	4·7	16·0	10·0	
	Degenerating	6·4	15·8	10·3	106
GP	Intact control	2380	1820	1490	
	Degenerating	1990	960	600	62
IP	Intact control	910	860	830	
	Degenerating	1740	1260	730	143

animals, thus confirming the previous finding of MAGEE and ROSSITER (1954). The specific radioactivity of the GPC in the degenerating nerves was markedly increased. The labelling of GPE also was increased in the sectioned nerves, as was that of the IP in two of the three animals studied at this time interval; the specific radioactivity of GPS was not changed significantly, and that of GP was decreased.

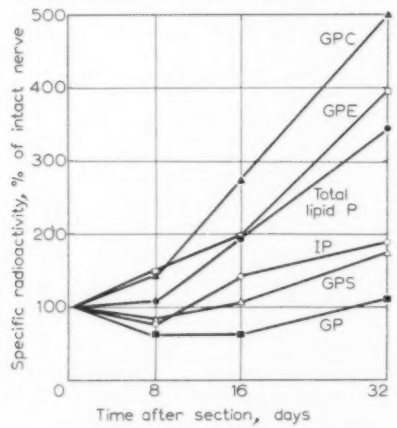

FIG. 1.—Effect of nerve section on the *in vitro* incorporation of inorganic ^{32}P into the phosphatides of cat sciatic nerve. GPC, –▲–▲–. GPE, –□–□–. GPS, –△–△–. GP, –■–■–. IP, –○–○–.

Fig. 1 shows the changes in the specific radioactivity of each of the phosphatides from nerves studied 8, 16 and 32 days after section. The mean change is expressed as a percentage of the specific radioactivity of the intact control nerves. It can be seen that, although the specific radioactivity of the total lipid P was only slightly increased 8 days after section, it increased sharply during the period from 8 to 32 days, to reach a value

greater than three times that of the control nerve by the end of 32 days. After nerve section, the specific radioactivity of the GPC and GPE increased with each successive period of degeneration, the effect being more marked with GPC. With the exception of the decrease in the labelling of GP at 8 and 16 days and the increase in the labelling of IP at 32 days, the values plotted in Fig. 1 for IP, GPS and GP are not significantly different from 100, i.e. there is no statistically significant difference between the means for the sectioned and the intact nerves.

TABLE 3.—EFFECT OF NERVE SECTION ON THE CONCENTRATIONS OF CERTAIN ACID-SOLUBLE PHOSPHORUS COMPOUNDS OF THE SCIATIC NERVE OF THE CAT

Concentration expressed as mg P/100 g wet weight ± S.E.M.

	No. of determinations	Intact control	Degenerating (8 days)	% of intact nerve	P
Inorganic P	4	6·15 ± 0·17	6·09 ± 0·28	99	>0·8
ATP	4	1·70 ± 0·21	2·18 ± 0·07	128	>0·05
ADP	4	1·84 ± 0·34	1·65 ± 0·08	90	>0·6
AMP	4	0·85 ± 0·12	1·31 ± 0·03	154	<0·05
	No. of determinations	Intact control	Degenerating (16 days)	% of intact nerve	P
Inorganic P	6	5·28 ± 0·24	5·37 ± 0·22	102	>0·7
ATP	6	1·51 ± 0·05	2·57 ± 0·12	170	<0·001
ADP	6	1·03 ± 0·09	0·72 ± 0·28	70	>0·3
AMP	6	0·79 ± 0·05	1·37 ± 0·05	173	<0·001

Table 3 shows that there was a significant increase in the concentration of AMP in the degenerating nerve at the end of the incubation period. This was true for nerves studied both 8 and 16 days after section. The mean value for the concentration of ATP also was greater for the degenerating nerves, both 8 and 16 days after section, but only the 16-days figures attained the conventional level of statistical significance. There was no significant change in the concentration of ADP.

Associated with these changes in the concentrations of the adenosine phosphates, there were few significant changes in specific radioactivity (Table 4). The specific radioactivity of the ADP was significantly decreased 8 days after section and still further decreased by 16 days. However, labelling of the nerve inorganic P, ATP and AMP was not significantly affected by the operation.

The values reported in Table 3 represent those reached after cat nerve had been incubated for 90 min under good metabolic conditions. Table 5 shows the concentration of the same phosphorus compounds in the sciatic nerve of the rat. In these experiments the nerves were not incubated. At 12 days after section the concentration of AMP was increased, as observed for the cat nerves at both 8 and 16 days after section. In the rat nerves there was also a slight, though statistically significant, increase in the concentration of inorganic P, not observed in the degenerating cat nerves. There were no significant changes in the concentration of either ATP or ADP. The values reported here for rat sciatic nerve are somewhat higher than those reported

by ABOOD and GERARD (1954) and those for ATP are a little lower than those reported by GREENGARD, BRINK and COLOWICK (1954) for the sciatic nerve of the frog. The latter workers also found no significant change in the concentraion of ATP during Wallerian degeneration.

DISCUSSION

The results indicate that the increase in the incorporation of inorganic ^{32}P into the phospholipid of metabolizing fragments of degenerating cat sciatic nerve is due largely to an increase in the specific radioactivity of phosphatidyl choline and phosphatidyl ethanolamine, as opposed to phosphatidyl serine and phosphatidic acid. The figures for inositol phosphatide are more equivocal.

The increase in the labelling of phosphatidyl choline, in particular, is of interest, since the available evidence indicates that phosphatidyl choline is not a myelin lipid (BRANTE, 1949; JOHNSON, MCNABB and ROSSITER, 1950; FOLCH, 1955; ROBINS, EYDT and SMITH, 1956). Presumably the phosphatidyl choline that is labelled is located in the Schwann cells, because at this time the axoplasm, which is rich in phosphatidyl choline (MCCOLL and ROSSITER, 1950), has almost completely disappeared. The increased labelling of a non-myelin lipid in the Schwann cell, which is certainly active metabolically (FOSTER, 1956; MAJNO and KARNOVSKY, 1958*a*), is discussed further in the following paper (PRITCHARD and ROSSITER, 1958).

It seems likely that the degenerating nerve has an increased ability to form phosphatidyl choline, for ROSSITER, MCLEOD and STRICKLAND (1957*a*) have shown that cytidine diphosphate choline labelled with ^{32}P is incorporated more rapidly into the phosphatidyl choline of homogenates prepared from rat sciatic nerves degenerating after section. This finding indicates an increase in the activity of an enzyme, phosphorylcholine-glyceride transferase, which is necessary for the formation of lecithin in both liver (KENNEDY and WEISS, 1956) and brain (ROSSITER, MCMURRAY and STRICKLAND, 1957*b*). However, in cat sciatic nerves studied 16 days after section there was no change in the activity of choline kinase, another enzyme concerned with lecithin synthesis (BERRY, MCPHERSON and ROSSITER, 1958). By 32 days the activity of this enzyme in degenerating nerves was less than that in intact controls.

The increase in the *in vitro* incorporation of inorganic ^{32}P into the phospholipid of degenerating cat sciatic nerve reported here, and previously (MAGEE and ROSSITER, 1954), could possibly be related to the increase in the concentration of ATP shown in Table 3. However, if this is the main reason for the increase in the lipid labelling, one might expect an increase in the labelling of ATP (not observed, Table 4) and a generalized increase in the labelling of all phosphatides. Also no increase in the concentration of ATP has been observed in degenerating nerve that has not been incubated (Table 5; GREENGARD *et al.*, 1954), yet *in vivo* there is an increased incorporation of inorganic ^{32}P into the phospholipid P (BODIAN and DZIEWIATKOWSKI, 1950; SAMUELS *et al.*, 1951).

Recently PORCELLATI (1958) reported that there was an increase in the concentration of free phosphorylethanolamine in sectioned rat nerves. If such an increase occurred in cat nerve, a decrease in the labelling of phosphatidyl ethanolamine from inorganic ^{32}P might be expected, because of the isotope dilution effects of the larger pool of free phosphorylethanolamine, which is an intermediate in the formation of phosphatidyl ethanolamine (KENNEDY and WEISS, 1956). Despite this, however, the labelling of phosphatidyl ethanolamine was increased in the sectioned nerves.

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TABLE 4.—EFFECT OF NERVE SECTION ON THE SPECIFIC RADIOACTIVITIES OF CERTAIN ACID-SOLUBLE PHOSPHORUS COMPOUNDS OF THE SCIATIC NERVE OF THE CAT

Specific radioactivity expressed as counts/min per μg P ± S.E.M.

	No. of determinations	Intact control	Degenerating (8 days)	% of intact nerve	P
Inorganic P	4	51,400 ± 110	49,700 ± 3100	97	>0·6
ATP	4	11,700 ± 830	11,700 ± 350	100	>0·9
ADP	4	7900 ± 260	6500 ± 170	82	<0·02
AMP	4	1470 ± 50	1420 ± 80	96	>0·2
	No. of determinations	Intact control	Degenerating (16 days)	% of intact nerve	P
Inorganic P	6	57,900 ± 900	56,400 ± 660	97	>0·2
ATP	6	9900 ± 230	9900 ± 190	100	>0·9
ADP	6	4400 ± 220	2500 ± 30	57	<0·001
AMP	6	930 ± 20	910 ± 20	97	>0·4

TABLE 5.—CONCENTRATIONS OF CERTAIN ACID-SOLUBLE PHOSPHORUS COMPOUNDS IN CONTROL AND DEGENERATING SCIATIC NERVES OF THE RAT

Period of degeneration, 12 days. Concentration expressed as mg P/100 g wet weight ± S.E.M.

	No. of determinations	Intact control	Degenerating	% of intact nerve	P
Inorganic P	8	21·9 ± 1·6	26·4 ± 2·4	121	<0·05
ATP	12	7·8 ± 1·2	7·6 ± 0·9	98	>0·8
ADP	12	6·9 ± 1·2	7·6 ± 1·4	111	>0·5
AMP	12	3·7 ± 0·3	5·3 ± 0·4	140	<0·01

As suggested previously (ROSSITER *et al.*, 1957*a*,*b*; 1958), the increased labelling of the myelin lipids in degenerating nerve may represent an increase in the capability of the nerve to remyelinate. This potential is realized, only if the degenerating distal segment is reinervated by a growing axon tip from the central stump. During the subsequent regeneration, the Schwann cells are undoubtedly capable of a high rate of myelin lipid formation. In this regard, it is noteworthy that the total activity (counts/min per 100 mg nerve) of nerves regenerating 96 days after a crushing injury was considerably greater than that of nerves degenerating for the same period of time after section (MAGEE and ROSSITER, 1954). Similarly, KLINE *et al.* (1958) showed that when fragments of regenerating cat sciatic nerve were incubated with ^{14}C-labelled acetate, there was a large increase in the specific radioactivity and the total activity of both phospholipid and non-esterified cholesterol.

SUMMARY

(1) Inorganic ^{32}P was incorporated into the phospholipid ATP, ADP and AMP of fragments of cat sciatic nerve respiring in a Krebs–Ringer medium containing glucose.

(2) In cat sciatic nerves degenerating 8, 16 and 32 days after section and incubated under similar conditions, the specific radioactivity of the phospholipid was increased. The increase was largely due to an increase in the labelling of phosphatidyl choline and phosphatidyl ethanolamine, as opposed to phosphatidyl serine and phosphatidic acid.

(3) In nerves studied 8 and 16 days after section there was an increase in the concentration of AMP and a decrease in the specific radioactivity of ADP. In nerves degenerating 16 days after section there was an increase in the concentration of ATP.

(4) In unincubated rat sciatic nerves degenerating 12 days after section, the concentration of AMP and inorganic P was increased, whereas there was no significant change in the concentration of ATP or ADP.

(5) The results are discussed in relation to chemical and histological changes known to occur during Wallerian degeneration.

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CHEMICAL STUDIES OF PERIPHERAL NERVE DURING WALLERIAN DEGENERATION—XI

IN VITRO INCORPORATION OF ^{14}C-LABELLED PRECURSORS INTO PHOSPHATIDES

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THE *in vitro* incorporation of certain isotopically labelled precursors into the lipids of respiring fragments of peripheral nerve is greatly increased in nerves undergoing Wallerian degeneration. References to the relevant literature are given in the preceding paper (MAGEE, BERRY, MAGEE and ROSSITER, 1958).

By suitably choosing the precursors, PRITCHARD (1958) showed that it was possible to label different parts of the glycerophosphatide molecule. For rat brain slices incubated *in vitro*, acetate-1-^{14}C labels the fatty acid portion, glycerol-1-^{14}C labels the glycerol portion, whereas choline-1:2-^{14}C, ethanolamine-1-^{14}C and serine-3-^{14}C each label the appropriate base moiety. It became of interest, therefore, to investigate the relative *in vitro* incorporation of each of these precursors into intact peripheral nerve and nerves degenerating after section.

Preliminary accounts of some of these experiments have appeared (ROSSITER, 1957; ROSSITER, PRITCHARD and STRICKLAND, 1958).

METHODS

The right sciatic nerve of a number of cats was sectioned. The details of the selection of animals, the operation, and the method of preparing the nerve for experiment were the same as those described in the previous paper (MAGEE *et al.*, 1958). The method of incubation of the nerve fragments with ^{14}C-labelled precursors, extraction, separation and hydrolysis of phospholipids, separation of hydrolysis products of individual phosphatides and counting were the same as those described by PRITCHARD (1958).

RESULTS

Intact nerves

Table 1 shows the specific radioactivity of the phospholipid from fragments of intact cat sciatic nerve that had been allowed to respire for 4 hr in the presence of acetate-1-^{14}C, glycerol-1-^{14}C, choline-1:2-^{14}C, ethanolamine-1:2-^{14}C, or serine-3-^{14}C. In the last column the corresponding figures are given for slices of rat brain that had been incubated under similar conditions, but for 3 hr only (PRITCHARD, 1958). In comparing the incorporation of the different precursors for both cat sciatic nerve and rat brain it should be borne in mind that for choline-1:2-^{14}C the added radioactivity was only 0·6 μC in 3 μmoles, compared with 2 μC in 3 μmoles for the other substrates. Also the serine was in the DL-form.

The relative order of activity for the fragments of cat sciatic nerve was the same as that observed for slices of rat brain, although the specific radioactivity of the lipids in the nerve was always considerably lower, even although the incubation time was

1 hr longer. This relative difference between cat nerve and rat brain was less for acetate-1-^{14}C and glycerol-1-^{14}C than for the other precursors. In this regard it is of interest to note that MAJNO and KARNOVSKY (1958*a*), who studied the relative *in vitro* incorporation of various ^{14}C-labelled precursors into the lipids of rat sciatic nerve and brain, found that acetate-1-^{14}C was the only precursor that did not give a

TABLE 1.—INCORPORATION OF ^{14}C-LABELLED PRECURSORS INTO THE PHOSPHOLIPID OF CAT SCIATIC NERVE AND RAT BRAIN

Cat nerve fragments and rat brain slices incubated at 37·5° in Krebs–Ringer bicarbonate, pH 7·4 containing 11 mM glucose. Incubation time, 4 hr (nerve fragments), 3 hr (brain slices). To each vessel was added 3 μmoles precursor containing 2 μc radioactivity, except for choline (0·6 μc).

^{14}C-labelled precursor	Cat nerve specific radioactivity (counts/min per μg lipid P)	Mean	Rat brain* specific radioactivity
Acetate-1-^{14}C	6, 7, 8, 9, 8, 8	7·5	22 ± 2 (8)
Glycerol-1-^{14}C	9, 17	13	54 ± 3 (19)
Choline-1:2-^{14}C	5, 4, 4, 5	4·5	74 ± 6 (10)
Ethanolamine-1:2-^{14}C	26, 23	24·5	315 ± 9 (2)
Serine-3-^{14}C	6, 5, 9, 9	7	91 ± 9 (7)

* Counts/min per μg lipid P ± standard error of mean. Figures in parentheses indicate the number of experiments.

higher specific radioactivity in brain than in nerve. However, the results of MAJNO and KARNOVSKY (1958*a*) are not strictly comparable with those reported here, because of differences in species, concentration of precursors, and the conditions of the incubation.

Nerve degenerating after section

Table 2 shows that the incorporation of the radioactivity of each of the precursors into phospholipid was considerably increased in nerves that had been allowed to degenerate for either 16 or 32 days after section. At 16 days the relative increase for ethanolamine-1:2-^{14}C and serine-3-^{14}C was greater than that observed for the other precursors. By 32 days, however, this difference had largely disappeared.

Since it is known that the concentration of phospholipid in a peripheral nerve decreases considerably during Wallerian degeneration (JOHNSON, MCNABB and ROSSITER, 1950, for references), the figures for specific radioactivity (counts/min per μg P) in the first two columns of Table 2 were converted into total radioactivity (counts/min per 100 mg intact nerve), as described by MAGEE and ROSSITER (1954). The data of LOGAN, MANNELL and ROSSITER (1952) for the concentration of lipid P in degenerating cat sciatic nerve were used for the calculation. The last two columns of Table 2 show that, with the exception of acetate-1-^{14}C and glycerol-1-^{14}C 16 days after section, the radioactivity in terms of counts/min per 100 mg nerve for all of the precursors and at both time intervals after section was considerably increased in the degenerating nerves.

The figures reported for specific radioactivity in Table 1 represent counts/min per μg P. However, the phospholipid fraction of cat sciatic nerve represents a mixture of

a number of different phosphatides. If the labelling of these phosphatides in cat nerve follows a pattern similar to that observed for rat brain (PRITCHARD, 1958), it is probable that the specific radioactivity of any phosphatide will depend upon both the

TABLE 2.—EFFECT OF NERVE SECTION ON THE INCORPORATION OF ^{14}C-LABELLED PRECURSORS INTO THE PHOSPHOLIPID OF THE SCIATIC NERVE OF THE CAT

Nerve fragments incubated as described in Table 1.
Each figure represents the average for four samples.

^{14}C-labelled precursor	* Specific radioactivity D/I		†Radioactivity D/I	
	16 days	32 days	16 days	32 days
Acetate-1-^{14}C	2·8	10·0	1·4	2·5
Glycerol-1-^{14}C	2·0	21·2	1·0	5·4
Choline-1:2-^{14}C	4·0	22·8	2·0	5·8
Ethanolamine-1:2-^{14}C	15·1	17·0	7·6	4·3
Serine-3-^{14}C	21·8	21·4	11·0	5·5

* Specific radioactivity $D/I = \frac{\text{counts/min per } \mu\text{g lipid P in degenerating nerve}}{\text{counts/min per } \mu\text{g lipid P in intact nerve}}$

† Radioactivity $D/I = \frac{\text{counts/min per 100 mg degenerating nerve}}{\text{counts/min per 100 mg intact nerve}}$

nature of the phosphatide, as was demonstrated when the radioactivity was derived from inorganic ^{32}P (MAGEE *et al.*, 1958), and the nature of the ^{14}C-labelled precursor used. Because of this, and because of the probability that in degenerating nerves the concentration of each phosphatide will be different from that found in intact nerves,

TABLE 3.—EFFECT OF NERVE SECTION ON THE INCORPORATION OF ^{14}C-LABELLED PRECURSORS INTO THE HYDROLYSIS PRODUCTS OF PHOSPHATIDES OF THE SCIATIC NERVE OF THE CAT

Nerve fragments incubated as described in Table 1. The phosphatide hydrolysis products were separated by the method of DAWSON (1954).

^{14}C-labelled precursor	Fraction isolated	*Specific radioactivity D/I	
		16 days	32 days
Choline-1:2-^{14}C	GPC	2·4	8·8
Ethanolamine-1:2-^{14}C	GPE	18·0	16·0
Serine-3-^{14}C	GPS	24·0	31·0

* Specific radioactivity $D/I = \frac{\text{counts/min per } \mu\text{g P in fraction isolated from degenerating nerve}}{\text{counts/min per } \mu\text{g P in fraction isolated from intact nerve}}$

the hydrolysis products of the labelled phosphatides in both degenerating and intact nerves were separated by the method of DAWSON (1954).

Table 3 shows that when choline-1:2-^{14}C, ethanolamine-1:2-^{14}C, or serine-3-^{14}C was the precursor, the respective specific radioactivity of glycerylphosphorylcholine (GPC), glycerylphosphorylethanolamine (GPE) or glycerylphosphorylserine (GPS) was greater in the degenerating nerves than in the intact control nerves, both 16 and

32 days after the nerve section. Since GPC, GPE and GPS are derived, respectively, from phosphatidyl choline (lecithin), phosphatidyl ethanolamine and phosphatidyl serine (DAWSON, 1954), it may be concluded with reasonable safety that the labelling of each of these phosphatides from the appropriate precursor is considerably increased in degenerating nerve.

Examination of the hydrolysis products of the labelled phospholipid showed that for both intact and degenerating cat nerve, as for rat brain slices (PRITCHARD, 1958), the radioactivity from each of the above three precursors was confined to the appropriate base. For example, the radioactivity of choline-1:2-^{14}C was found almost exclusively in GPC, with none in GPE or GPS. When the GPC was further hydrolysed, all of the radioactivity was recovered in the choline, with none in the glycerol.

When ethanolamine-1:2-^{14}C was the precursor, the increases in the specific radioactivity after nerve section were of the same order regardless of whether the counts were referred to the lipid P of the nerve (Table 2) or to the phosphorus of the isolated GPE (Table 3). This similarity is due to the fact that phosphatidyl ethanolamine disappears from the degenerating nerve at a rate comparable to that for the disappearance of total lipid P. A similar situation was found to exist when the radioactivity was derived from serine-3-^{14}C. However, when choline-1:2-^{14}C was the precursor, the relative increases in the degenerating nerve were less when the specific radioactivity was referred to the phosphorus of GPC (Table 3) than when it was referred to the total lipid P of the nerve (Table 2). This is probably due to the fact that there is a small loss of phosphatidyl choline (and hence GPC) as the nerve degenerates (JOHNSON, MCNABB and ROSSITER, 1949; BRANTE, 1949; MCCAMEN and ROBINS, 1958).

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DISCUSSION

The results indicate that when slices of brain or fragments of peripheral nerve tissue are allowed to respire in a suitable medium containing the appropriate ^{14}C-labelled precursors, the phosphatides present in the brain or nerve are labelled, thus confirming the results of PRITCHARD (1958) and MAJNO and KARNOVSKY (1958*a*). When nerves degenerating after previous section are incubated under similar conditions, there is an increase in the labelling of the phospholipids. This was demonstrated previously when the radioactivity was derived from either inorganic ^{32}P (MAGEE and ROSSITER, 1954; MAGEE *et al.*, 1958) or acetate-1-^{14}C (KLINE, MAGEE, PRITCHARD and ROSSITER, 1958; MAJNO and KARNOVSKY, 1958*b*).

With acetate-1-^{14}C as precursor, the increase in specific radioactivity of the phospholipid was quite small in nerves that had been allowed to degenerate for 16 days before the nerve was removed for incubation with the labelled substrate. By 32 days the increase for acetate-1-^{14}C was considerably greater than at 16 days, but it was smaller than that observed for the other precursors that were studied. For acetate-1-^{14}C the effect of time of degeneration on the labelling of the phospholipids appears to be different from that observed for the other precursors. MAJNO and KARNOVSKY (1958*b*) reported that for rat sciatic nerve degenerating after section, the incorporation of acetate-1-^{14}C into phospholipid was actually decreased during the first week after the operation, followed by a subsequent increase. Such a biphasic response was not observed by MAGEE and ROSSITER (1954) when inorganic ^{32}P was the precursor. In the present study no observations are reported at time intervals shorter than 16 days from the operation.

For glycerol-1-^{14}C and choline-1:2-^{14}C the increases in specific radioactivity, like those observed for acetate-1-^{14}C, were quite small at 16 days. However, by 32 days the increases were of the same order as those observed for ethanolamine-1:2-^{14}C and serine-3-^{14}C. It may thus be seen that when a nerve degenerates after section there is an increase in the incorporation of radioactivity of ^{14}C-labelled phosphatide precursors into the phospholipid, but that the time course of this increase is not the same for each of the precursors.

The metabolic events described above are no doubt intimately related to the anatomical and cellular changes occurring during the degeneration. Previously it was suggested that the increase in the incorporation of acetate-1-^{14}C into the cholesterol and phospholipid of degenerating nerve might indicate that these nerves are 'more amply provided with the metabolic machinery necessary for the formation of myelin lipids' (KLINE *et al.*, 1958). Since the work of GEREN (1954) has shown that in a peripheral nerve the Schwann cell is responsible for the formation of the myelin sheath, it is tempting to suggest that the increase in the *in vitro* lipid labelling from acetate-1-^{14}C (KLINE *et al.*, 1958; MAJNO and KARNOVSKY, 1958*b*), inorganic ^{32}P (MAGEE and ROSSITER, 1954; MAGEE *et al.*, 1958) and other ^{14}C-labelled precursors (this paper) is related to the proliferation of Schwann cells and that the increase in labelling represents an increase in the ability of the nerves to form myelin, should a suitable opportunity present itself (ROSSITER *et al.*, 1958). Such an opportunity occurs when a nerve fibre that has degenerated after an injury receives a growing axon tip from the central stump. Under these conditions the nerve becomes remyelinated as a result of the metabolic activity of the Schwann cells.

However, the above interpretation, which possibly may be true in a general sense, is complicated by two experimental findings. The first is the difference in the time course observed for the increase in labelling for each of the precursors, mentioned above. The second is that the increase in labelling is not confined to myelin lipids. It seems reasonable to assume that phosphatidyl choline quantitatively is not a major myelin lipid (BRANTE, 1949; JOHNSON *et al.*, 1950; FOLCH, 1955; ROBINS, EYDT and SMITH, 1956), yet there is a great increase in the labelling of GPC, which is derived from phosphatidyl choline, either when inorganic ^{32}P (MAGEE *et al.*, 1958) or choline-1:2-^{14}C (this paper) is the precursor. Another anomaly is the labelling of GPS, which is derived from phosphatidyl serine. In degenerating nerve there is a greatly increased labelling of GPS from serine-3-^{14}C (this paper), but no increase in the labelling of GPS from inorganic ^{32}P (MAGEE *et al.*, 1958). Thus in the degenerating nerve the *in vitro* incorporation of one precursor into phosphatidyl serine is increased, but there is no change in the incorporation of another. Phosphatidyl serine may be a myelin lipid (BRANTE, 1949; ROBINS *et al.*, 1956), although this point is far from clear (FOLCH, 1955).

These findings serve to illustrate the complexity of the situation in degenerating nerve and the difficulties involved in the interpretation of existing data. There can be no doubt but that there is an increase in the *in vitro* labelling of certain of the myelin lipids in degenerating nerve, yet at the same time there is an increase in labelling of lipids that are not thought to be major constituents of the myelin sheath. The increase in the incorporation of labelled precursors into the myelin lipids may represent an increase in the ability of the nerve to form myelin. However, the increase in the incorporation of labelled precursors into non-myelin lipids, like the increase in the

labelling of ribonucleic acid and other phosphorus-containing compounds previously reported (MAGEE and ROSSITER, 1954), represents a new pattern of activity for the nerve as a whole, possibly associated with the increase in the number (ABERCROMBIE and JOHNSON, 1946) and metabolic activity (FOSTER, 1956; MAJNO and KARNOVSKY, 1958*b*) of the sheath cells.

SUMMARY

(1) The phospholipid of the sciatic nerve of the cat was labelled when fragments of the nerve were incubated in a Krebs–Ringer medium containing glucose and either acetate-1-^{14}C, glycerol-1-^{14}C, choline-1:2-^{14}C, ethanolamine-1:2-^{14}C, or serine-3-^{14}C.

(2) In nerves degenerating either 16 or 32 days after section and incubated under similar conditions, the specific radioactivity of the phospholipid was increased when the radioactivity was derived from each of the above ^{14}C-labelled precursors.

(3) The increase in the labelling was observed both for phosphatides thought to be components of the myelin sheath and for phosphatides thought not to be myelin sheath components. The time course of the increase in labelling was not the same for each phosphatide.

(4) The results are discussed in relation to chemical and histological changes known to occur during Wallerian degeneration.

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THE HISTOCHEMICAL IDENTIFICATION OF MYELIN PHOSPHOGLYCERIDES BY THEIR FERRIC HYDROXAMATES

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HISTOCHEMISTRY suffers from a lack of methods for identifying different types of lipid. There is, for example, no reliable staining method for distinguishing phospholipid from glycolipid (PEARSE, 1953). Phospholipid *in vitro* gives a strong reaction with BAKER's (1946) acid haematin method, but there is some doubt whether glycolipid is also faintly stained (BAKER, 1946; 1947; CASSELMAN, 1952). This method also has the disadvantage that it is laborious in practice. Likewise FEYRTER's (1936) metachromatic method probably stains both types of lipid.

In search of a specific colour-reaction for the histochemical detection of phospholipid in myelin we have adapted the ferric hydroxamate reaction used by RAPPORT and ALONZO (1955) for the estimation of phosphatides. We have found that this method when carried out in aqueous medium demonstrates phosphoglycerides. Short-chain (C_2—C_4) triglycerides and certain non-physiological esters such as the Tweens react *in vitro* but may be ignored in the histochemical application. Sphingomyelin, glycosphingosides, long-chain triglycerides, cholesterol and cholesterol esters do not react under these conditions.

METHODS AND RESULTS

Hydroxamate test

Reagents. An alkaline solution of hydroxylamine was prepared by mixing equal parts of 3 N-sodium hydroxide and 5 per cent hydroxylamine hydrochloride. An acid iron solution was prepared as a 0·6 per cent solution of ferric chloride in 0·2 N-hydrochloric acid.

In the standard test lipid smears or tissue-sections were treated with the alkaline hydroxylamine solution for 20 min. After rinsing in tap-water the preparations were placed in the acid iron solution for a further period of 20 min. In preliminary trials it was found that the test could not be improved by the substitution of ferric chloride by ferric perchlorate as recommended by HILL (1946), while copper, cobalt and nickel hydroxamates were too feebly coloured to be seen in tissue-sections. The intensity of colour obtained after incubation at 37° and 60° was no more than that found at room temperature. The intensity of colour of the ferric hydroxamate complex was not improved by attempted post-coupling with hydroquinone, catechol, sulphosalicylic acid and $\alpha\alpha'$-dipyridyl or by treatment with potassium ferricyanide, potassium ferrocyanide or ammonium sulphide. For these reasons we confined our investigations to the standard method described above.

Investigations in vitro. Representative lipids were spread as streaks or smears on glass slides: as far as possible the thickness of each smear was the same. In some cases where the lipid did not easily adhere to the slide it was covered with a layer of celloidin. These preparations were then subjected to the standard hydroxamate test. To increase the physical accessibility of the reagents to the lipids, the test was also carried out in an 80 per cent ethanol solution both at room temperature and at 60°. The lipids used and the colour reactions seen are recorded in Table 1.

TABLE 1.—THE FERRIC HYDROXAMATE REACTION WITH LIPIDS *in vitro*

Lipid	Solvent		
	Water (15°C)	Ethanol (15°C)	Ethanol (60°C)
Lecithin*	Orange-red		
Lecithin-Sphingomyelin fraction†	Orange-red		
Cephalin fraction†	Orange-red		
Sphingomyelin‡	—	—	—
Cerebroside fraction†	—	—	—
Ganglioside§	—	—	—
Polyoxyethylene sorbitan monostearate (Tween 60)	Orange-red		
Triacetin (C_2)	Red	Red	Red
Tributyrin (C_4)	Red	Red	Red
Trivalerin (C_5)	—	Red	Red
Tricaproin (C_6)	—	Red	Red
Triolein (C_{18})	—	—	Red
Tristearin (C_{18})	—	—	Red
Cholesterol	—	—	—
Cholesterol acetate	—	Pink	Red
Cholesterol oleate	—	—	Red
Cholesterol stearate	—	—	Red

* Prepared from fresh eggs by chromatographic separation according to the method of RHODES and LEA (1957).
† Prepared from whole rabbit brain by chromatographic separation.
‡ Prepared by alkaline hydrolysis of the lecithin-sphingomyelin fraction of rabbit brain.
§ Prepared according to the method of FOLCH, ASCOLI, LEES, MEATH and LE BARON (1951).

The blockading techniques described below were applied before the hydroxamate test in order to confirm that the active chemical group responsible for the reaction was in fact the acyl or carboxylic acid ester link.

For unsaturated groups (—CH=CH—). The lipid preparations were treated with saturated aqueous bromine for 4 hr. In no case did this diminish the subsequent hydroxamate reaction.

For acetal bonds (*plasmalogens*). The lipids were treated with both normal hydrochloric acid and with saturated mercuric chloride for 1 hr. Again this did not prevent the hydroxamate reaction.

For the carboxylic acid ester link. Each lipid was hydrolysed in 0·2 N-sodium hydroxide at room temperature for 30 min. The hydroxamate reaction of the lipid became negative after such alkaline hydrolysis.

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Investigations on tissue sections

The tissues listed in Table 2 were fixed in 4 per cent formaldehyde-saline and were cut at 10–15 μ on the freezing microtome. Unfixed tissues were also cut at 5 μ on the cryostat. The sections were then stained by the same standard hydroxamate method as used with the lipid smears *in vitro*. For convenience in microscopy the sections were counter-stained for 15 sec in a 2 per cent solution of alcian blue (I.C.I.) in 2 N-sulphuric acid.

TABLE 2.—THE FERRIC HYDROXAMATE REACTION WITH LIPIDS IN TISSUE SECTIONS

Tissue		Intensity of reaction
(A) *Human*		
CNS	myelin	+++
	degenerating myelin (Marchi positive)	—
	axon	—
	glial fibres	—
	ground substance	—
	neuronal lipochrome	− to ++
PNS	myelin	+++
	axon	—
Fatty liver:	fat globules	—
Adrenal cortex:	zona glomerulosa	—
	zona fasciculata	—
	zona reticulosa	+
Aorta:	atheromatous plaque	—
Erythrocyte membrane		++
Connective-tissue:	fat globules	—
(B) *Chick*		
CNS	myelin at hatching	+
	12 days old	++
	57 days old	+++

The results obtained are presented in Table 2. The colour seen in those tissue lipids which reacted was a reddish orange: it was somewhat transparent but sufficiently intense for satisfactory localization.

Preliminary bromination, acid hydrolysis, alkaline hydrolysis and oxidation by mercuric chloride were carried out with the tissue-sections in the same way as with the lipid smears. Only alkaline hydrolysis was effective in preventing the reaction.

DISCUSSION

The ferric hydroxamate reaction (Fig. 1) demonstrates the presence of the carboxylic acid ester group (HILL, 1946; HESTRIN, 1949). These ester groups are also

shown by the histochemical test since the reaction is abolished by preliminary alkaline hydrolysis. Unsaturated groups and acetal bonds are not responsible for the reaction as destruction of these groups has no effect on the test. Furthermore sphingolipids fail to react, probably because the fatty acid side chain is attached by an amide rather than an ester link.

Rapport and Alonzo (1955), who used the test in aqueous solution, observed that certain phospholipids reacted with the hydroxylamine reagent; these lipids were lecithin, lysolecithin, phosphatidylserine, phosphatidylethanolamine and 'cardiolipin'. Phospholipids form a polar surface to water and it has been shown that such hydrophilic lipids can be stained by aqueous solutions of certain dyes and reagents while hydrophobic lipids, such as the triglycerides, are not accessible to aqueous reagents and, correspondingly, are not stained by polar dyes (ADAMS, 1958). It follows that

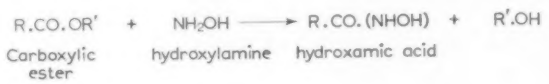

FIG. 1.

the negative hydroxamate reaction of hydrophobic lipids, in an aqueous medium, might reasonably be attributed to their physical inaccessability to the reagents. To support this hypothesis we found that the intensely hydrophilic Tween 60 reacted in an aqueous solution of the reagents, while hydrophobic lipids were stained when the test was carried out in boiling ethanol. Furthermore some of the shorter-chain triglycerides formed an intermediate group, for they failed to react in aqueous medium but were stained at room temperature in an ethanolic solution of the reagents (see Table 1 and BAUER and HIRSCH, 1949).

When a hydrophobic lipid (cholesterol oleate) was mixed with a phosphoglyceride (lecithin) the hydroxamate reaction was suppressed if more than 50 per cent of the mixture was hydrophobic lipid. This is in accord with DIXON's (1958) concept of micellar polar (hydrophilic) lipid changing into globular (hydrophobic) lipid as a result of the deposition of cholesterol or other organotropic lipids on the micelle. The conversion *in vivo* of hydrophilic lipid into hydrophobic may account for CAIN's (1950) observation that mixtures of lipids do not necessarily present the same staining properties as the individual lipids of such a mixture.

It was concluded that phosphoglycerides are the only lipids likely to be stained in histological sections by the hydroxamate reaction, for these lipids are water-attractive and, unlike the hydrophilic sphingolipids, they possess an acyl ester link. The investigations on tissue-sections support this conclusion, for a strong reaction was found in myelin and in the erythrocyte membrane: both these structures contain large amounts of phosphoglycerides (BRANTE, 1949; FOLCH-PI, 1955; TURNER, 1958). Lipid storage granules in Niemann–Pick's disease (sphingomyelin), Gaucher's disease (cerebroside) and Tay–Sach's disease (ganglioside) all gave a negative hydroxamate reaction: this was expected, for the stored lipid in each of these diseases is sphingolipid rather than phosphoglyceride. Degenerating myelin, connective tissue fat and other sudanophilic lipids (see Table 2) did not react; this again was expected since these lipids are all stained by the Marchi test (ADAMS, 1959) and are accordingly hydrophobic.

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It is hoped that this ferric hydroxamate reaction will prove of value for the histochemical demonstration of phosphoglyceride, particularly for the study of the chemical processes in myelination.

SUMMARY

(1) The ferric hydroxamate reaction for the carboxylic acid ester group has been adapted for the histochemical demonstration of phosphoglyceride.

(2) Phosphoglycerides, on account of their hydrophilia, react with an aqueous solution of hydroxylamine; fats and other hydrophobic lipids only react in hot alcoholic solutions of the reagents; sphingolipids do not react. Thus, when the reaction is applied in aqueous rather than alcoholic solution, phosphoglycerides are the only naturally-occurring tissue lipids stained.

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SELECTIVE DEPLETION OF NORADRENALINE AND 5-HYDROXYTRYPTAMINE FROM RAT BRAIN AND INTESTINE BY RAUWOLFIA ALKALOIDS*

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IT is a property common to reserpine and some other Rauwolfia alkaloids that they deplete noradrenaline and 5-hydroxytryptamine (5-HT) from various tissues. That the release of these amines does not run parallel, at least in rat brain, was demonstrated by PAASONEN and DEWS (1958) by using raunescine. This alkaloid decreased the concentration of noradrenaline faster and probably more completely than that of 5-HT. This was contrary to the findings of BRODIE and co-workers (BRODIE, OLIN, KUNTZMAN and SHORE, 1957; SHORE and BRODIE, 1957) showing a parallel depletion of these two substances in the rabbit brain-stem by reserpine.

This paper contains evidence that the noradrenaline concentration is lowered before 5-HT also by reserpine, and that this selective depletion is especially clear in the intestine. A special decrease pattern of these amines, quite different from that produced by raunescine and reserpine, has been demonstrated by using yohimbine.

METHODS

Rats of both sexes weighing from 100 to 180 g were used. Experiments were made at room temperature (20–22°C). In most cases the following procedure was followed. A group of from 6 to 8 animals of the same sex and approximately the same size were employed. Two rats formed one experimental unit, i.e. each value obtained is a mean from 2 animals. The rats were killed by decapitation. The brains (excluding the cerebellum) were dissected out as far as a level just caudal to the *area postrema*. The tissue was cut sagitally into two equal parts, one part from each brain being used for the 5-HT and the other for the noradrenaline estimation. About 1 g from the intestine (duodenum and upper jejunum) of each rat was taken. The loose contents were wiped off and one-half of the combined tissue was used for the 5-HT and the other half for the noradrenaline determination. In each experiment 2 rats served as controls.

For the estimation of 5-HT tissues were ground with saline and sand, extracted with acetone, and the lipids removed with petroleum ether according to AMIN *et al.* (1954). For the determination of noradrenaline the brains were ground with 10% trichloroacetic acid and the intestine with 5% trichloroacetic acid and sand. The procedure suggested by VON EULER (1956) was used with minor changes (PAASONEN and KRAYER, 1958).

The amines were assayed biologically by using the rat stomach method of VANE (1957) for 5-HT and the cat blood pressure method for noradrenaline. The same combination of drugs as used by KÄRKI (1956) was administered to the cat before the noradrenaline assay.

In addition to the above mentioned extraction procedure a modification of the method used for catecholamine estimation by SHORE and OLIN (1958) was employed. For biological assay we found it better to extract tissues first with 90% acetone instead of butanol. After addition of heptane the aqueous phase still contained some acetone which was evaporated *in vacuo* at 35°. This extraction has been quite useful for 5-HT estimation from the rat brain and intestine, recoveries from added 5-HT being 80 per cent or more. It cannot be used for estimations of noradrenaline in the intestine

* A communication presenting these results has been made at the Third Acta Endocrinologica Congress, Leiden, 16–19 June 1958 (PAASONEN and KÄRKI, 1958).

or on the cat's blood pressure because of inhibitory activity of the extracts. Of 5 cats 2 gave unsatisfactory responses to the brain extracts as well. The results obtained by using this 'acetone–heptane method' are not included in Table 1.

Raunescine and reserpine were dissolved in glacial acetic acid and the solution diluted so that the concentration of acetic acid was not in excess of 2 per cent. All drugs were administered intraperitoneally and control experiments were made using the vehicle alone. Yohimbine hydrochloride (MERCK, DARMSTADT) was diluted with water. 5-HT was used as creatinine sulphate and noradrenaline as bitartrate. All doses refer to the weight of the base.

RESULTS

The results are presented in Table 1. The standard deviation in control groups was rather large (alimentary, seasonal, age differences?), whereas values obtained under strictly identical conditions were very similar. Therefore it was thought necessary to calculate each figure also as a percentage from the control value obtained at the same time. The means only of these are presented in the table.

Brains

Raunescine. In the brains the results with a dose of 5 mg/kg were similar to those reported earlier (PAASONEN and DEWS, 1958): less than 50 per cent of 5-HT was lost as compared to about 80 per cent loss of noradrenaline. Half of this dose seemed to lower the noradrenaline content but not the 5-HT.

Reserpine. There was an indication that 0·5 and 1·25 mg/kg of reserpine decreased the noradrenaline level more than the 5-HT. This was evident with a dose of 2·5 mg/kg when practically no noradrenaline was detectable, but about half of the 5-HT was still present.

Yohimbine. This alkaloid was injected in two or three doses. No decrease in 5-HT followed, but the two highest doses lowered the noradrenaline content by some 40 per cent. Seven further experiments, where only noradrenaline was determined, confirmed this observation. Within 4 hr 20 mg/kg of yohimbine lowered the concentration of noradrenaline to 60 per cent (range 26–79) of the control values.

Intestine

Raunescine. Intestinal 5-HT was not influenced even with the highest dose to any significant degree. Noradrenaline, however, was depleted within 4 hr, nothing being found after 2·5 and 5·0 mg/kg and less than half of the control amount after 0·5 mg/kg of raunescine.

Reserpine. The result agreed with that obtained with raunescine. It was evident that some of the 5-HT was lost, but an increase of the dose did not make this effect any more marked (or more rapid). A dose of 0·1 mg/kg reduced the amount of noradrenaline to a just detectable level and, after higher doses, no activity was found in any of the samples tested.

Yohimbine. As in brain tissue, there was no depletion of 5-HT. In contrast to the two other alkaloids, yohimbine did not produce any change of noradrenaline level in this tissue.

Symptoms in the animals.

In doses able to release noradrenaline from the brain, raunescine and reserpine produced typical sedation, eyelid ptosis and piloerection. A dose which did not lower the 5-HT value, such as 2·5 mg/kg of raunescine, had a distinctive tranquillizing effect.

TABLE 1—EFFECTS OF RAUNESCINE, RESERPINE AND YOHIMBINE ON THE 5-HYDROXYTRYPTAMINE AND NORADRENALINE CONTENT OF RAT BRAIN AND INTESTINE. THE AMINE CONTENT IS GIVEN IN mμg/g WET WEIGHT OF TISSUE. DRUGS WERE INJECTED INTRAPERITONEALLY 4 HR BEFORE KILLING THE ANIMALS

Drug	Dose (mg/kg)	5-Hydroxytryptamine	Mean ± S.D.	%*	Noradrenaline	Mean ± S.D.	%*
			Brain				
Controls	—	(n† = 14)	216 ± 57	100	(n† = 14)	202 ± 45	100
Raunescine	0·5	365, 220, 182	256	119	197, 165, 245	202	97
Raunescine	2·5	162, 190, 215	189	107	118, 190, 110	140	62
Raunescine	5·0	183, 155	169	62	37, 30	59	21
Reserpine	0·5	225, 166	196	77	127, 130	129	67
Reserpine	1·25	112, 142, 185, 62	125	80	193, 112, 35, 43	96	49
Reserpine	2·5	97, 78, 130	102	46	<39‡, 12, 37	⩽23	⩽17
Reserpine	12·5	19	19	15	12	12	5
Yohimbine§	10·0	150, 175	163	83	156, 126	141	106
Yohimbine§	20·0	280	280	116	57, 98, 145	100	62
Yohimbine§	30·0	235, 218, 257, 191	225	113	83, 104, 133, 155	119	55
			Intestine				
Controls	—	(n† = 13)	2790 ± 1130	100	(n† = 13)	211 ± 41	100
Raunescine	0·5	3100, 1950, 2980	2680	112	<37, 76, 97	⩽70	⩽33
Raunescine	2·5	1690, 3090	2390	125	<42, <34	0	0
Raunescine	5·0	2890, 2800	2850	90	<49, <32	0	0
Reserpine	0·1	1940	1940	100	20	20	8
Reserpine	0·5	2200, 2400	2300	102	<30 <20	0	0
Reserpine	1·25	1500, 3000, 2140, 1910	2140	65	<44, <64, <14, <14	0	0
Reserpine	2·5	1200, 2100	1650	68	<22, <15	0	0
Reserpine	12·5	2100	2100	63	<12	0	0
Yohimbine§	10·0	1240, 1790	1520	85	240, 150	195	98
Yohimbine§	20·0	3950	3950	97	164	164	71
Yohimbine§	30·0	1040, 1000, 5600, 4800	3100	89	240, 150, 187, 220	199	90

* The mean percent value. The percentages were calculated from the control figures of the same day.
† Number of animals.
‡ Not detectable(<).
§ In divided doses. More data on yohimbine are given in the text.

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The two highest yohimbine doses have to be considered as sublethal. With 20, and particularly 30, mg/kg of this alkaloid the rats were sedated to some extent and they occasionally had tonic–clonic convulsions. There was no eyelid ptosis and the animals were usually cold to touch. In order to find out whether the noradrenaline depletion was due to the hypothermia, 2 pairs of rats were kept at an environmental temperature of 37° during the period of yohimbine (20 mg/kg) action. The brain noradrenaline values of the treated animals were 96 and 143 mμg/g as compared with 220 and 262 mμg/g in rats kept as controls at room temperature. Another experiment was made where 4 pairs of rats were kept at a temperature of about 16° and another 4 pairs at about 26° during the yohimbine (20 mg/kg) action. No obvious difference was found between the brain noradrenaline values of the two groups.

Acetone–heptane method

By this method values for 5-HT were obtained which were 50–70 per cent higher than by the procedure commonly employed in this study. The relative effects of raunescine and reserpine, however, remained the same. In two experiments, one with 5 mg/kg of raunescine and one with 2·5 mg/kg of reserpine, where the brain noradrenaline estimation was satisfactory, the results were in keeping with those given in the table.

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DISCUSSION

It is evident that in the rat raunescine and reserpine in suitable doses are able to lower the noradrenaline content in brain and especially in intestine more completely or faster than the 5-HT content. This study has shown that the dose of 5 mg/kg of raunescine used previously by PAASONEN and DEWS (1958) was a fortunate choice for discovering this difference. It cannot be concluded whether there is only a quantitative difference in the action of these two alkaloids. A dose of 2·5 mg/kg seemed to be the best dose of reserpine to show the selective action in an experiment of 4 hours. In 2 out of 4 experiments the effect of 1·25 mg/kg clearly affected noradrenaline more than 5-HT. For some reason the sensitivity to reserpine seemed to vary from time to time.

In the intestine the smallest doses were effective in reducing the noradrenaline concentration. A dose of 0·1 mg/kg of reserpine was as effective or more effective than doses five times higher of raunescine. It is known that 5-HT is not readily depleted from intestine, neither is its metabolism influenced by iproniazid in this tissue as it is in brain (PLETSCHER, 1956). The effect of 1·25 and 12·5 mg/kg of reserpine seemed to be the same during the 4 hour test period employed.

BRODIE and collaborators (SHORE and BRODIE, 1957) have shown that after the administration of 5 mg/kg of reserpine intravenously to rabbits, the level of noradrenaline in the brain stem declines at the same rate and to the same degree as that of 5-HT. Also a comparison of the time course and dose-response curves of the loss of these amines indicated that the effects on the two substances are in fact superimposable. BRODIE *et al.* used different doses, and the explanation of this discrepancy cannot be found in the dosage. The possibility that the different mode of administration gave higher initial blood reserpine levels in the rabbit experiments than in our rats injected intraperitoneally has to be considered. Species differences are also possible, especially in the light of the previous experiments, which show that there are clear differences in the reaction to reserpine of adrenaline and noradrenaline in the peripheral sympathetic system between one type of test animal and another (MUSCHOLL and

VOGT, 1958). We are in the process of finding out whether the difference found in rat whole brain also appears in the brain-stem of this animal and whether rat brain is able to take up temporarily some of the 5-HT set free from other tissues. It is of interest to note the experiments of PLETSCHER (1958) and PLETSCHER *et al.* (1958), where, after pretreatment with iproniazid, reserpine and tetrabenazine lowered the catechol amine content of the brain in rats and rabbits more than the 5-HT content.

We are so far unaware of the exact localization of the 5-HT and noradrenaline in the brain tissue. Ultracentrifugal experiments indicate that they are located in intracellular granules (BLASCHKO, 1957), but this does not mean they are present in the same granules or even in the same cells.

The situation in the intestinal mucosa is not the same. The most probable site of 5-HT here is the enterochromaffin cell system (ERSPAMER, 1957). It is generally assumed that noradrenaline is located in sympathetic nerve fibres (VON EULER, 1956). So the localization of these two amines in this organ is most probably quite different. The heart noradrenaline has been reported to be extremely sensitive to reserpine action (CARLSSON *et al.*, 1957). In the rat intraperitoneal reserpine administration (0·5 mg/kg) caused a loss of about 75 per cent of the noradrenaline from heart tissue (unpublished observation). A fifth of that dose of reserpine depleted the noradrenaline more completely from the intestine in the same time.

That yohimbine did not reduce 5-HT values is in keeping with the postulate of BRODIE *et al.* (1956) that only the tranquillizing Rauwolfia alkaloids are able to do this. As far as noradrenaline is concerned, however, there was about 50 per cent depletion from the brain tissue in our experiments. Yohimbine may be one addition to the group of drugs found by VOGT (1954) to be able to deplete the level of hypothalamic sympathin (adrenaline and noradrenaline) and to stimulate the adrenomedullary secretion by way of impulses through the splanchnic nerves. None of VOGT's active substances, when tested on the 5-HT of dog hypothalamus and caudate nucleus, were found to lower the content of this amine (PAASONEN and VOGT, 1956). The influence of yohimbine on noradrenaline is fundamentally different from that of raunescine and reserpine, because there is no clear decrease in intestinal noradrenaline values. Our experiments at high environmental temperatures give evidence against the possibility that the depletion of brain noradrenaline is due to the hypothermia produced by yohimbine.

From the results presented it is impossible to draw any conclusions concerning the mode of Rauwolfia action. It might be emphasized that there was a clear sedative action after a dose of raunescine, which was able to lower the noradrenaline but not the 5-HT content in the brain. If we assume that the cerebral noradrenaline has a transmitter role like that in the peripheral sympathetic system (VOGT, 1954), these findings lend some support to the view that Rauwolfia alkaloids exert their tranquillizing effects through the release of noradrenaline.

SUMMARY

Raunescine, reserpine and yohimbine were injected intraperitoneally into rats and the animals were killed 4 hours after the drug administration. Both 5-hydroxytryptamine (5-HT) and noradrenaline from brain and intestinal tissue were analysed by bioassay.

In suitable doses raunescine (5 mg/kg) and reserpine (2·5 mg/kg) are able to

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deplete about 80 per cent of the noradrenaline content as compared with a 40–55 per cent loss of 5-HT in the brain. In the intestine this difference is very clear. Doses of 0·1 mg/kg of reserpine and 0·5 mg/kg of raunescine deplete most of the noradrenaline, but doses from ten to a hundred times higher do not lower the 5-HT content in this tissue by more than about 35 per cent.

Yohimbine (20–30 mg/kg) leaves 5-HT intact in both tissues. In these doses it lowers brain noradrenaline by no more than 50 per cent. It does not reduce the noradrenaline content of intestine. Experiments carried out at elevated environmental temperatures indicate that the effect on brain noradrenaline is not due to the hypothermia that yohimbine is likely to produce at room temperature.

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AMINO ACID AND PROTEIN METABOLISM OF THE BRAIN—V

TURNOVER OF LEUCINE IN MOUSE TISSUES

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INTRODUCTION

THE study of the synthesis of proteins in the brain occupied several laboratories in recent years (GAITONDE and RICHTER, 1956; PALLADIN and VERTAIMER, 1955; LAJTHA, FURST and WAELSCH, 1957*b*; FURST, LAJTHA and WAELSCH, 1958). It was shown that the brain takes up and incorporates into its proteins a smaller amount of labelled amino acid administered intravenously or intraperitoneally than do some tissues, such as liver, but more than others, such as muscle (LAJTHA, FURST, GERSTEIN and WAELSCH, 1957*a*). The recent findings pointed out the fact that slow incorporation is not due mainly to the inactivity of brain proteins but to the restriction the blood–brain barrier places on the entrance of free amino acids into the brain from the circulation (LAJTHA, 1958; FRIEDBERG, TARVER and GREENBERG, 1948). The portion of labelled amino acids which entered the brain was incorporated into brain proteins rapidly.

In our laboratory we found, with the aid of [^{14}C] lysine, a relatively rapid flux of this amino acid from the pool of free amino acid in the brain into brain proteins. Furthermore, it was found that the calculated half-life time of proteins depended on the duration of the experiment (LAJTHA *et al.*, 1957*a*). Shorter experiments resulted in shorter turnover times. This finding was interpreted as showing the metabolic heterogeneity of the proteins, and it was concluded that experiments of short duration would measure mainly the protein fractions with a high rate of turnover, while in experiments of long duration the turnover of proteins of lower metabolic activity would be measured.

Although rates of incorporation of one amino acid are usually taken as a measure of the rate of protein turnover, it has to be ascertained whether or not we are really measuring the turnover of the protein or that of the introduction of the amino acid into and removal from the peptide linkage at a rate different from that of the synthesis or degradation of the whole protein. Therefore, measurement of turnover rates with more than one amino acid is necessary in order to make sure that turnover of the protein bound amino acid really represents the turnover of the protein. Consequently, the turnover rates of the proteins of liver, brain and muscle measured previously with [^{14}C] lysine were estimated with [^{14}C] leucine.

As in the previous experiments, the entrance of uniformly labelled [^{14}C] leucine from the circulating plasma into the nonprotein fraction of brain, liver, and muscle of adult and newborn mice and the incorporation of the free amino acids of the

respective tissues into the tissue proteins was estimated. The findings with leucine support out previous results with lysine and demonstrate again the metabolic heterogeneity of the organ proteins, the rapid passage of the free amino acid from plasma into a pool of free amino acid of the organ, and the larger portion of rapidly metabolizing proteins in young, as opposed to adult animals.

EXPERIMENTAL

The methods for the preparation of the protein and nonprotein fractions, hydrolysis of the proteins and determination of radioactivity and leucine content were the same as used previously for lysine (LAJTHA *et al.*, 1957*a*). No correction was made for radioactivity present in other compounds than leucine. In a separate set of experiments leucine was isolated from the hydrolysed proteins as the bromotoluene sulphonate salt (STEIN, MOORE, STAMM, CHOU and BERGMANN, 1942). It was found that in these short term experiments the organ proteins contained from 2 to 8 per cent of radioactivity which was not due to radioleucine.

Briefly, the organs were frozen after removal from the animal and extracted with 5% trichloroacetic acid (TCA), and the TCA extract (nonprotein nitrogen fraction = n.p.n.) and the protein fraction were worked up separately. After removal of the TCA from the n.p.n., aliquots of this solution were plated on aluminium planchets and their radioactivity determined in a Nuclear Chicago gas flow counter, and the leucine content of another aliquot was determined microbiologically using the method of HENDERSON and SNELL (1948). Proteins, after the extraction of nucleic acids and lipids and drying, were counted as powders and their leucine content determined microbiologically after hydrolysis with 6 N-HCl.

C_3H mice were used for all the experiments. Adult animals (about 100 days old) were injected intravenously in the tail vein, newborn animals intraperitoneally. Uniformly labelled L-[^{14}C] leucine was used, 8 μC/μmole (Nuclear Chicago Corp.); 0·2 μC was injected in each adult animal in 0·1 ml volume. Newborn animals were injected with a quarter of this dose. Six animals were pooled and their organs worked up together for each time point. For the experiments with newborn mice ten animals were pooled.

Flux of leucine from plasma free leucine to organ free leucine and the flux from free to protein bound leucine in the organs were calculated according to REINER (1953); for easier visualization of the results flux values were computed into half-life times (compare LAJTHA *et al.*, 1957*a*).

RESULTS

The leucine content of mouse tissues is shown in Table 1 with standard deviation.

TABLE 1.—LEUCINE CONTENT OF MOUSE TISSUES

	Proteins (μg/leucine per mg dry protein)		Free amino acids (μg/leucine per g fresh tissue)	
	Adult	Newborn	Adult	Newborn
Plasma	94 ± 9	126 ± 12	17 ± 3	18 ± 4·8
Brain	82 ± 10	91 ± 9·8	9·6 ± 1·4	20 ± 6·4
Liver	92 ± 9	99 ± 13	42 ± 5	30 ± 6·1
Muscle	84 ± 11	94 ± 13	23 ± 3	37 ± 6·7

The free leucine injected in the blood rapidly leaves the circulation and is taken up by the organs in adult as well as in newborn animals (Figs. 1(a) and 1(b)). In adult mice, brain initially takes up leucine at a rate comparable to liver but slows down later. Because of the very rapid changes and because of variations from animal to animal, calculations of free amino acid turnover rates are only approximate. Since the data

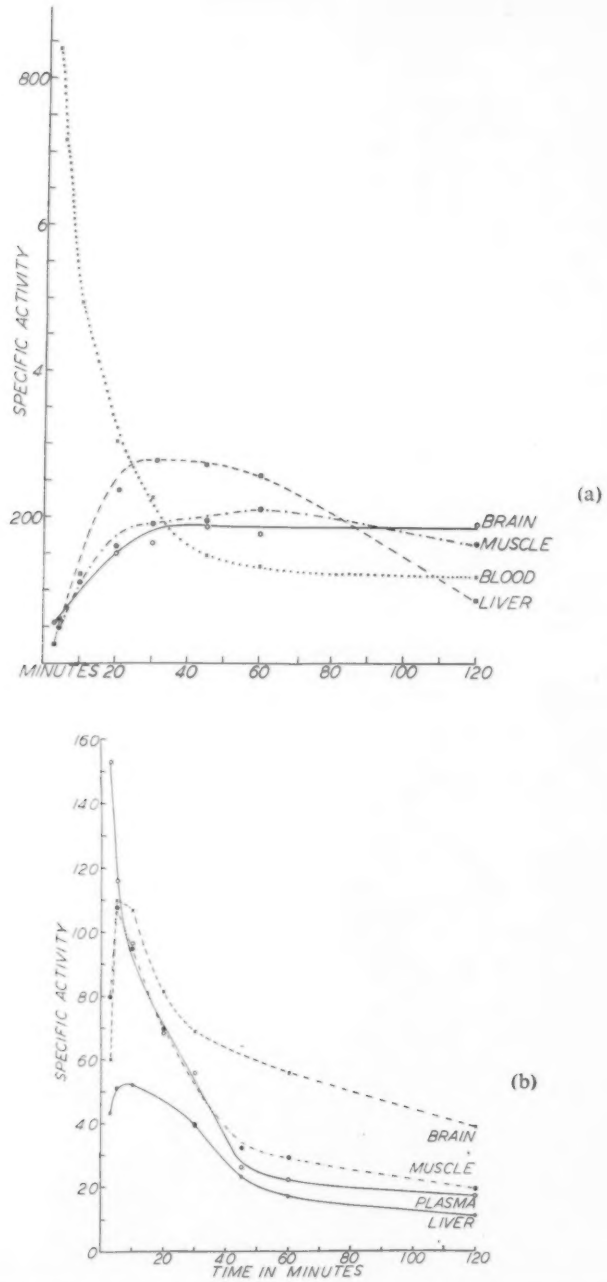

FIG. 1.—Change of specific activity (count/minute per μg leucine) of free leucine with time. (a) = newborn mice. (b) = adult mice.

are semiquantitative, no exact value is claimed for the half-life times and the figures are given only to show that the exchange is rapid. The results of such calculations are presented in Table 2, which shows that the half-life time of leucine in the mouse tissues studied is probably not more than 1 hr—a value in the range of that found for free lysine of mouse tissues.

TABLE 2.—FREE LEUCINE TUROVER RATES IN ADULT MICE

Brain		Liver		Muscle	
F	$t_{1/2}$	F	$t_{1/2}$	F	$t_{1/2}$
3·0	3	3·1	9	2·8	7
1·5	6	0·6	45	2·1	9
0·6	10	1·7	13	0·5	28
0·1	55	3·0	11	1·4	10
0·1	65	5·8	4		
0·1	62	3·5	9		

Flux = F = μg leucine/g fresh tissue/min. Half-life time = $t_{1/2}$ in minutes.

While the liver (and probably the muscle) data vary independently of the duration of the experiment, in brain the flux decreases with increasing experimental time. It is possible that this is an indication that the free amino acids in the organs are not in a homogeneous pool. An indication that the free glutamic acid and glutamine pools of the organs are heterogeneous was presented in a previous paper (LAJTHA, BERL and WAELSCH, 1959).

The free leucine in the organs subsequently is incorporated into protein bound leucine. The changes in specific activities of the protein bound leucine are shown in Figs. 2(a) and 2(b). The isotope is incorporated at the highest rate into liver protein. Plasma proteins also are labelled rapidly, but this is not shown as well in short term experiments. Plasma proteins show a lag period of about 20 min, which is probably the time it takes for newly synthesized plasma proteins in the liver to reach the circulating plasma.

Proteins in brain are labelled at a lower rate than in liver, but faster than in muscle and in a few minutes significant quantities of radioleucine are to be found in brain proteins.

From the knowledge of the change in time in the specific activities of a precursor and a product, flux rate, and from this turnover time can be calculated (REINER, 1953). This calculation was used on the data on leucine presented here. As in other calculations of this type, in this method of arriving at flux rates the usual assumptions are made that the free leucine in the organ is in a homogeneous pool, and that it is the sole precursor of the protein bound leucine, and that during the experimental period no significant protein breakdown occurs (Table 3). It can be seen that the half-life time of the organ proteins is not a constant number but depends on the duration of the experiment, and increases with increasing time intervals between the administering of the [^{14}C] leucine and the sacrificing of the animal, a finding in accord with that made when [^{14}C] lysine was used in the turnover studies.

TABLE 3.—HALF-LIFE TIME ($t_{1/2}$) OF PROTEINS AND FLUX (F) OF LEUCINE FROM N.P.N. INTO PROTEIN

Time after administration (min)	Brain				Liver				Muscle			
	Adult		Newborn		Adult		Newborn		Adult		Newborn	
	$t_{1/2}$	F	$t_{1/2}$	F	$t_{1/2}$	F	$t_{1/2}$	F	$t_{1/2}$	F	$t_{1/2}$	F
3	3·8	8·8	0·7	71	0·4	94	0·2	280	5·1	6·8	0·9	58
5	8·1	4·6	1·2	31	0·9	50	0·4	110	7·1	6·1	1·5	23
10	11	3·3	1·7	29	1·3	34	0·7	72	14	3·0	2·0	23
20	15	2·7	2·4	19	2·4	20	1·4	40	24	1·7	2·9	18
30	20	2·2	3·0	16	3·9	13	2·0	23	28	1·8	3·2	15
45	24	2·1	3·3	13	5·7	9·2	4·0	13	30	1·5	4·1	11
60	27	1·3	4·1	9·8			4·7	8	34	1·0	5·0	9·4

Flux = F = μg leucine/g dry protein per min. Half-life time = $t_{1/2}$ in days.

DISCUSSION

The present results with [^{14}C] leucine closely parallel our previous results with [^{14}C] lysine. Both amino acids when injected into the circulating blood are distributed throughout the organism rapidly, and the half-life time of the free amino acids in the organs can be measured in minutes. Because of the rapid changes in plasma and the variations from animal to animal (for measuring different time points different animals have to be sacrificed) such values are only approximate, but the results show that the half-life time of free amino acids in the organs is short and for brain or liver the half-life time of leucine and lysine is less than 1 hour.

Amino acids from the free amino acid pool in the organs are subsequently incorporated into organ proteins. The pattern of incorporation with leucine shows the metabolic heterogeneity of the organ proteins just as well as the lysine incorporation studies did. The calculated protein half-life time, or the flux into proteins, depends on the duration of the experiment. This is due most probably to the presence of protein fractions with widely different turnover rates. In short term experiments the fraction which is incorporating amino acids rapidly would influence results more, and in experiments of longer duration such an active fraction might turn over several times and would influence the calculations of the turnover rates less. This fact points out the very approximate absolute value of the 'half-life time' number of the proteins of such a complex organ as brain. Most probably the turnover rate spectrum of brain proteins is very wide and one could find fractions which are considerably more active than the average value actually measured, and fractions which are less active or completely inactive. From our studies and those of others on the incorporation of proteins of brain areas or brain cell fractions, it is likely that some of the proteins have turnover rates of a few hours or less.

The analogous finding obtained with two amino acids as to rates of incorporation of lysine and leucine into protein and the dependence of the calculated half-life times for the experimental period strengthen greatly the assumption that we are measuring turnover rates of proteins and not of one particular amino acid which attaches itself to or is detached from tissue protein at a rate different from that of the turnover.

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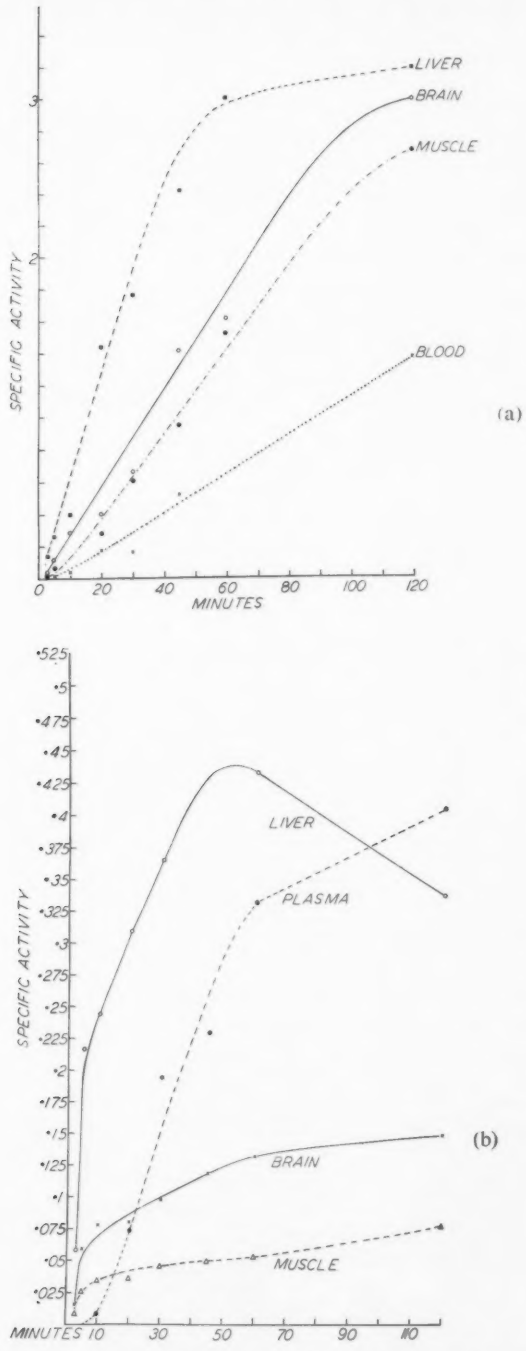

FIG. 2.—Change of specific activity (count/minute per μg leucine) of protein bound leucine with time (a) = newborn mice. (b) = adult mice.

The flux of leucine from the pool of free amino acids into the protein shows a decrease and the derived values for half-life time show an increase with increasing experimental period—the values for the flux in newborns being at all time periods higher than those found in the adult animal. These data appear to be at variance with those found when lysine was used as the isotopic amino acid, where the flux values at the shortest experimental period were no higher in young than in adult animals, but where in young, unlike adults, no significant decrease of flux was found at a longer period. This finding led to the conclusion that the slowly metabolized proteins were not present in young, while the rapidly metabolized proteins were turned over at a rate not considerably faster than in the adult animal. It is of interest to note that in the lysine experiments the animals, although immature, were approximately 8 days of age, while in the leucine experiments, new born animals were used. In the newborn animals the increment of newly deposited protein is considerably greater than in the 8 day old mouse and it is probably this fact which results in the appearance of proteins of lower turnover rate than in the 8 day old or adult animal. The average rate of protein synthesis in the 8 day old animal is apparently already equal to that of the average turnover rate of the fractions most rapidly metabolized in the adult animal while, on the other hand, slowly metabolized proteins do not comprise a considerable portion of the organ proteins in the young. In the rapdily growing tissue of the newborn animal, the rate of synthesis is higher than that of the protein with the highest turnover rate found in the adult tissue, while the slower fractions in the newborn are in the range of the values found for the 8 day old animals.

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SUMMARY

(1) The flux of leucine into mouse organs was measured after the injection of uniformly labelled L-leucine.

(2) Plasma leucine exchanged rapidly with the free leucine in brain, liver and muscle with an estimated half-life time of organ free leucine less than 1 hour.

(3) The leucine was incorporated from the free amino acid into protein bound leucine in the organs; the flux in liver was highest, followed by brain, then muscle.

(4) The half-life time of organ proteins was not constant but increased with increasing experimental time period. This was interpreted as a result of the metabolic heterogeneity of organ proteins.

(5) Tissue proteins turned over more rapidly in newborn than in adult animals but showed similar metabolic heterogeneity as adult tissue proteins.

The results which confirm our previous findings with lysine are compared with these data.

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